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Identification and Characterization of Genes in Hybrid Poplar (*Populus trichocarpa* x *P. deltoides*) Upregulated by Mechanical Wounding and Herbivory by the Forest Tent Caterpillar (*Malacosoma disstria*).

By

Joseph John Patton ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Identification and Characterization of Genes in Hybrid Poplar (*Populus trichocarpa* x *deltoides*) Upregulated by Mechanical Wounding and Herbivory by the Forest Tent Caterpillar (*Malacosoma disstria*) submitted by Joseph John Patton in partial fulfillment of the requirements for the degree of Master's of Science in Molecular Biology and Genetics.

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Abstract

In hybrid poplar (*Populus trichocarpa* x *P. deltoides*), mechanical wounding, FTC herbivory, and methyl jasmonate are known to induce expression of polyphenol oxidase (PPO) and trypsin inhibitor (TI). To identify novel wound-responsive genes, a suppressive subtractive hybridization (SSH) library was constructed from young leaves showing strong systemic-induction of PPO and TI after wounding. Macro-arrays were differentially screened using ^{32}P-radiolabelled cDNA representing total mRNA from mechanically wounded, FTC browsed, and control leaves. Wound-induced cDNAs were sequenced and their predicted peptide sequences were used to query databases to predict their function. Induction locally and systemically was confirmed in poplar leaves after mechanical wounding. Herbivory was compared to mechanical wounding and the chemical elicitors methyl jasmonate and salicylic acid. Unwounded poplar tissues were examined to explore developmental gene expression of the wound-induced genes. The results presented in this thesis have expanded the number of known wound-induced genes in the poplar system.

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ABBREVIATIONS

AFLP	amplified fragment length polymorphism
AOS	allene oxide synthase
ANS	anthocyanidin synthase
BLAST	basic local alignment search tool
BSP	bark storage protein
BTH	benzothiadiazole
CAB	chlorophyll *ab* binding protein
cDNA	complementary DNA
CHS	chalcone synthase
4CL	4-coumarateCoA ligase
12,13-EOT	9Z, 11E,15Z,13S,12R-12,13-epoxy-9,11,15-octadecatrienoic acid
DFR	dihydroflavonol reductase
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequence tag
FACs	fatty acid conjugates
FTC	forest tent caterpillar
GST	glutathione-S-transferase
h	hours
6-HCH	6-Hydroxy-2-cyclohexenone
13(S)-HPOT	9Z,11E, 15Z, 13S)-13-hydroperoxy-9,11,15-octadecatrienoic acid
% ID	percent identity

JA	jasmonic acid
LA	alpha-linolenic acid
LAR	leucoanthocyanidin reductase
LNP	lectin-binding phosphohydrolase
LOX	lipoxygenase
LTP	Lipid transfer protein
MeJa	methyl-jasmonate
MOPS	3-(*N*-morpholino)-propanesulfonic acid
OPC-8:0	3-oxo-2(2'(Z)-pentenyl)-cyclopentane-1-octanoic acid
OPDA	12-oxo-10,15(Z)-octadecatrienoic acid
OPR	12-oxo-phytodienoic acid reductase
PAL	phenylalanine ammonia-lyase
PCR	polymerase chain reaction
pfam	protein families
PHI-BLAST	Pattern Hit Initiated BLAST
PI	proteinase inhibitor
PLA	phospholipase A
PPO	polyphenol oxidase
PSI-BLAST	Position Specific Iterated BLAST
% SIM	percent similarity
SSC	sodium chloride/sodium citrate
SSH	suppression subtractive hybridization
SSPE	sodium chloride/sodium phosphate/EDTA

TI	trypsin inhibitor
UV	ultraviolet
volicitin	N-17-hydroxylinolenoyl-L-glutamine
VSP	vegetative storage protein

CHAPTER 1

INTRODUCTION

A. INTRODUCTION

The major objective of this thesis is to identify novel genes from hybrid poplar (*Populus trichocarpa x P. deltoides*) whose expression is induced by forest tent caterpillar (*Malacosoma disstria*; FTC) or mechanical wounding. This will provide insight into the defense mechanisms of poplar against insect herbivory, and will be accomplished by differential cDNA screening of a FTC-induced systemic poplar leaf subtracted library. Gene expression after mechanical wounding and FTC herbivory will be compared to that in unwounded control leaves, and macroarrays will be used to identify strongly induced genes. These induced genes can then be characterized and their expression studied further.

This chapter reviews the literature relevant to this thesis. Specifically, it introduces poplar as a model for woody plant biology, provides an overview of induced defenses in poplar and other plants, discusses differences between mechanical wounding and insect herbivory, and discusses common methods for identifying differentially expressed genes.

B. BIOLOGY OF *POPULUS*

B-1 Poplar as a Model System for Woody Plant Biology

The genus *Populus* includes a number of economically significant species, which includes both poplars and aspens. This diverse genus harbors a rich source of variation in anatomy, morphology, phenology, physiology, and response to biotic and abiotic stress with much of this variation under moderate to strong genetic control (Farmer, 1996). Artificial *Populus* hybrids can be synthesized with relative ease and

are grown in large-scale plantations in both North America and Europe. Poplars are known to be hosts for many herbivorous insects and pathogens (Whitman *et al.*, 1996) and like conventional agricultural crops, plantations consisting of trees of similar age and genotype are susceptible to outbreaks of insect pests. Greater knowledge of how poplars respond to defoliating herbivores will provide a basis for the understanding of woody plant defense mechanisms as well as provide novel genes for the genetic engineering or selection of tree genotypes with superior insect resistance.

Some aspects of tree biology are easily studied in very tractable species such as *Arabidopsis* whereas other aspects cannot be studied in annual plants. Characteristics such as the extensive formation of wood from secondary xylem, seasonal (re)allocation of nutrients, perennial growth habit, and juvenile-mature phase change are more specific to woody species. The great lifespan and large size of many trees alone poses challenges to studying these problems. Variation in genotype and the inability to control the environment makes the problem of studying interactions at the molecular level virtually impossible in the field, but are amenable in greenhouse-grown tree seedlings and saplings. *Populus* has become a preferred model system for studying problems in woody plants due to its rapid growth, ease of clonal propagation, tractability to genetic transformation, genome size and the continuing accumulation of genomic data.

B-2 Distribution, Classification and Hybridization of *Populus*

The genus *Populus* L. is a member of the Salicaceae which, together with the Flacourtiaceae and 29 other families, have been placed under the Malpighiales, in the recent cladistic analysis of the angiosperms (The Angiosperm Phylogeny Group, 1998). Many *Populus* species occupy habitats of riverine floodplains where they form a key component of riparian forests (Braatne *et al.*, 1996) and are capable of rapidly invading disturbed sites. *Populus*, a genus of deciduous trees, comprises aspens, poplars and cottonwoods, having a wide natural distribution in the Northern Hemisphere and a small representation in tropical Africa. Many of these species have extensive distribution ranges. *P. tremuloides* and *P. tremula* span entire continents whereas *P. monticola* and *P. ilicifolia* are among the few that are geographically confined. One of the useful characteristics of poplar is the ease of sexual propagation and interspecific hybridization. Various classifications have been suggested, the most recent recognizing 29 species that are grouped under six separate sections (Eckenwalder, 1996). Those within the same section can be crossed quite easily and will do so in nature when the opportunity arises. All species share the same chromosome number (2n = 38) and their hybrids are fertile (Whitman *et al.*, 1996). *Populus* species have wind-pollinated dioecious flowers that produce large amounts of pollen and small cotton-tufted seeds that are dispersed by wind and water in early summer. Collected pollen can be stored for several years and, in the spring, it can be used in combination with detached branches to generate the desired parental crosses. Pollination can yield hundreds of seeds per detached branch within 4 to 8 weeks.

Seeds germinate within 24 hours and give rise to 1-2 m tall seedlings by the end of the same year.

B-3 Growth and Propagation of *Populus*

Poplar's premier asset as a model system is the ease with which it can be vegetatively propagated. This allows for the replication of experiments in time and space without the confounding effects of genetic variation. Clonal propagation permits the side-by-side growth of multiple generations of a pedigree for comparison. It also allows the gains attained by breeding or transgenic manipulation to be maintained indefinitely. Vegetative propagation facilitates the rapid bulking of material from a single individual to numbers required for field trials, while foregoing generation times required for sexual reproduction. In addition, the use of clones allows destructive sampling, the sharing of materials among laboratories, and the accumulation of knowledge on selected clone genotypes. It is because of this ease of propagation and the rapid growth rates of *Populus spp.* that hybrid poplar are being grown at a commercial scale under intensive culture in order to meet the growing demand for wood fiber (Fenning and Gershenzon, 2002).

An aggressive approach to poplar domestication by combining intensive genetic manipulation with intensive culture in remarkably short rotations has resulted in biomass production figures that are accordingly high. Specific hybrid clones from crosses between select parents of *P. deltoides* and *P. trichocarpa* demonstrate hybrid vigor and are not only superior to their parents (Scarascia-Mugnozza *et al.*, 1997) but are considered among the fastest growing trees in the temperate zone (Stettler *et al.*, 1996). Under 6-8 year rotations, production rates with hybrid poplar can be as high

as 16-30 Mg/ha/yr of dry woody biomass (Riemenschneider *et al.*, 2001; Zsuffa *et al.*, 1996), comparable to the biomass produced by row crops such as corn. Hybrid vigor combined with intense regulation of irrigated water and nutrient distribution accounts for the incredible growth rates. Longer rotations of 15-25 years are used to produce larger dimension logs. This strategy allows producers access to such diverse markets of value-added products as plywood, block board, oriented strand board, laminated beams, and lumber. In addition to harnessing hybrid vigor, research into the manipulation of tree characteristics through the introduction of transgenes has focused on four main areas: herbicide resistance, wood qualities, qualities relevant to bioremediation, and most relevant to this thesis, insect and pathogen resistance.

B-4 *Populus* and Transgenic Technologies

As with many agricultural plants, *Populus* is not limited to traditional breeding methods. The ease of creating transgenic *Populus* is unmatched by any other forest tree. *Populus* species have been transformed using microprojectiles (Devantier *et al.*, 1993) and *Agrobacterium* mediated transformation (Han, *et al.*, 1997; Confalonieri, *et al.*, 1997; Tzfira *et al.*, 1997; Tzfira *et al.*, 1996; Tsai *et al.*, 1995; Confalonieri *et al.*, 1994). Several poplar genotypes have been identified as amenable for genetic transformation, however, many other genotypes are more resistant (Whitman *et al.*, 1996). Using hybrid clones that are amenable to *Agrobacterium* mediated transformation, such as the hybrid aspen (*P. alba* x *tremula*) clone 717-1B4, transgenic trees can be efficiently generated within 6-10 months (Jouanin *et al.*, 1993). These modern molecular techniques allow us to operate at a larger biological

scale than in traditional breeding and draw from an immense gene pool beyond the boundaries of a single genus. Analysis using heterologous probes and transgenic promoter analysis across a wide phyletic space reveals a remarkable commonality among plant species. For example, probes from male-sterility mutants of *Arabidopsis* and *Antirrhinum* (snapdragon) have shown that black cottonwood has a corresponding region in its genome, and conserved tissue expression in the male anthers (Sheppard *et al.*, 2000). LEAFY, a gene from *Arabidopsis* involved in flower-meristem identity, is sufficient to induce precocious flowering in both *Arabidopsis* and the heterologous species aspen when constitutively expressed (Weigel and Nilsson, 1995). Other examples of heterologous transgenes showing conserved expression in poplar are the rice homeobox gene OSH1 (Mohri *et al.*, 1999) and a cinnamyl alcohol dehydrogenase promoter-GUS fusion from eucalyptus (Hawkins *et al.*, 1997). Furthermore, the conserved promoter expression patterns of different poplar genes in tobacco have been demonstrated several times (Gray-Mitsumune *et al.*, 1999; Hollick and Gordon, 1995; Clarke *et al.*, 1994; Hollick and Gordon, 1993). In summary, it would appear that poplars and herbaceous plants have conserved many promoter elements. This means that because many promoter elements are conserved, progress in other model plant systems in regards to spatial or temporal gene expression may, in many cases, be imported directly into poplar.

B-5 The *Populus* Genome

Populus has natural populations distributed world wide having adaptations to diverse conditions as well as prominent genetic polymorphisms in local populations.

Whereas polyploid genomes and other duplication events can create large gene families, smaller genomes simplify many molecular techniques, such as gene cloning and Southern blotting. Large gene families can result in cross hybridization during Northern and Southern analysis, mixed products in PCR reactions, and other problems for techniques that rely on sequence similarity. Poplar has a small genome [haploid genome size of 550 million base pairs (bp)] which is only four times larger than the genome of the model plant *Arabidopsis*, and 40 times smaller than the genomes of conifers such as loblolly pine (Bradshaw and Stettler, 1993). In addition, the *Populus* model is gathering a number of genomics related resources. Expressed sequence tag (EST) databases of modest size are in the public domain (Sterky *et al.* 1998). The physical:genetic distance ratio for poplar is close to 200 kb/centiMorgan, almost identical to *Arabidopsis*, making *Populus* an attractive target for map-based positional cloning of genes. In addition to linkage maps that show the location of genetic markers in relation to one another molecular linkage maps have been constructed for poplar (Liu and Furnier 1993, Bradshaw *et al.*, 1994). Linkage maps have been made with a variety of marker types, including allozymes, RFLPs, RAPDs, AFLPs, and microsatellites (Liu and Furnier, 1993; Bradshaw *et al.*, 1994; Cervera *et al.*, 1996; Frewen *et al.*, 2000). Currently, specific markers for traits such as growth, form, phenology, and disease resistance have been located (Bradshaw and Stettler, 1995; Villar *et al.*, 1996). Tracking of these markers in a selective breeding program can significantly increase its precision and efficiency and is practiced in the breeding of many agricultural crops.

Since *Populus spp.* has many of the key characteristics common to other herbaceous model systems, the accumulation of genomic data will allow tree breeders to profit from the cumulative knowledge of poplar morphology, physiology, and anatomy, unmatched by any other forest tree (Hinckley, 1996). *Populus spp.* are both ecologically and economically important throughout much of the world and are subjected to severe defoliation by a number of insect herbivores that cause damage to both plantations and vast tracts of wild *Populus* stands. Therefore, the combination of economic and ecological significance, severe periodic pest outbreaks, and model plant characteristics provide a unique opportunity to study insect-plant interactions in the woody perennial *Populus spp.* Specifically, in this thesis, the interaction between forest tent caterpillars and a poplar hybrid (*Populus deltoides* x *P. trichocarpa*) (hybrid H11-11) will be discussed.

C. WOUND-INDUCTION, HERBIVORY, AND PLANT RESPONSES

C-1 General Plant Responses

Plant defenses primarily fall into two categories: constitutive and induced. Constitutive defenses are expressed in specific tissues developmentally and may consist of physical structures such as thorns or toxic chemicals. To prevent the investment of resources in defenses that may not be required, plants also have defenses that are inducible. When the plant perceives tissue damage, it will induce defenses to reduce further herbivory or to prevent infection by opportunistic pathogens. In many cases, wounding induces defense not only in the damaged tissue but also in undamaged (systemic) tissue, presumably via a mobile wound signal. The

proteins encoded by wound-inducible genes may play a number of roles: participating in defense signaling pathways, repairing damaged tissues, responding to dehydration caused by cellular decompartmentalization, producing antinutritive or toxic compounds to inhibit pathogen or herbivore growth, or the mobilization of nutrients and adjusting plant metabolism to the imposed nutritional demands or other unknown functions. Although wound damage caused by different events may superficially be similar, some plants have demonstrated the ability to distinguish between different types of wounding events. At first glance, wounding caused by insect herbivores of plant tissue is caused by piercing or crushing damage and should be perceived as mechanical damage, but may in fact be perceived differently (Reymond *et al.*, 2000). Components of insect saliva (or other insect-derived chemicals) may be perceived by the plant which may then specifically modify the wound response (Mattiacci *et al.*, 1995; Alborn *et al.*, 1997; Korth and Dixon, 1997; Pohnert *et al.*, 1999; Halitschke *et al.*, 2001).

C-2 Systemic Induction and Systemin in Tomato

Much of what is known about defense related signaling stems from work on herbivore defense in tomato plants. When an insect attacks a plant, plants release localized and systemic signals that result in the induction of genes directed to healing of the damaged tissues and to the activation of defenses to repel the herbivore or pathogen. The induction of defense genes, such as antinutritive proteinase inhibitors, may not be limited to the wounded tissue and may include unwounded systemic tissues. Many structurally different molecules play regulatory roles in wound

signaling. Elicitors of local wound response include chitin fragments, cell wall degrading enzymes and fragments of the cell wall such as oligosaccharides and oligogalacturonides (Bishop *et al.*, 1984; Gundlach *et al.*, 1992; Doares *et al.*, 1995; Rojo *et al.*, 1999; Norman-Setterblad *et al.*, 2000). In addition to these local signals, the wound response is also controlled by plant hormones that include jasmonates (Farmer and Ryan, 1990), abscisic acid (Pena-Cortes *et al.*, 1989) and ethylene (O'Donnell *et al.*, 1996). A search for transported signal molecule involved in the systemic induction in unwounded leaves on wounded plants led to the identification of an 18-amino-acid polypeptide in tomato, named systemin (Figure 1-1). In tomato, the systemin polypeptide is active in femtomolar amounts and is nearly 1 million times more effective in inducing protease inhibitor genes than the earlier proposed signal, oligogalacturonide fragments (Schaller and Ryan, 1995).

The precursor protein of systemin was first cloned from a tomato leaf cDNA library and encodes a protein of 200 amino acids, with the systemin sequence located near its C-terminus (McGurl *et al.*, 1992). Systemin is believed to be transported through the phloem and has transport characteristics that closely resemble those of sucrose transport (Narvaez-Vasquez *et al.*, 1995). Once systemin has been translocated to the target cells the wound stimulus is transduced by the octadecanoid pathway or other signaling pathways including H_2O_2 (Stennis 1998; Orozco-Cardenas *et al.*, 2001). Ultimately, defense genes are transcriptionally activated and defense proteins produced.

Figure 1-1: Systemic Wound- and Herbivore-specific Signals

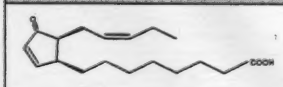
Examples of systemic wound- and herbivore-specific signals demonstrated to elicit either direct or indirect defenses in plants as adapted from Kessler and Baldwin (2002). Systemins are polypetide systemic wound signals from solanaceous plants, such as tomato (Tom Sys) and tobacco (Tob Sys I and II), which activate the octadecanoid pathway but are also inducible by various oxylipins (Pearce *et al.*, 2001; Ryan, 2000). Oxylipins, as illustrated by the octadecanoids, 12-oxophytodienoic acid (12-OPDA), jasmonic acid, and methyl jasmonate, elicit defense gene expression, numerous secondary metabolites, and insect resistance (Creelman and Mullet, 1997). Herbivore-specific elicitors have been identified in insect oral secretions and oviposition fluids, the two fluids that commonly come into contact with the wounded plant tissue. *Manduca* and *Spodoptera exigua* oral secretions contain the class of fatty acid-amino acid conjugates (FACs) found to actively elicit plant volatile organic compounds (VOCs), which function as indirect defenses by attracting parasitoids (Halittschke *et al.*, 2001; Turlings and Benrey, 1998). *Pieris brassicae* oral secretion contains the enzyme ß-glucosidase that also elicits VOC emission (Mattiacci *et al.,* 1995) to attract parasitic wasps. The novel class of elicitors from cowpea weevil oviposition fluid (bruchins) elicits neoplasmic tissue formation in peas, which expels the oviposited egg from the pea leaf tissue (Doss *et al.*, 2000).

Systemic wound signals

Systemins

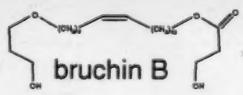
Tomato systemin	$^{+}$AVQSKPPSKRDPPKMQTD-
Tobacco systemin I	$^{+}$RGANLPOOSOASSOOSKE-
Tobacco systemin II	$^{+}$NRKPLSOOSOKPADGQRP-

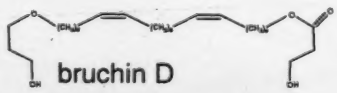
Octadecanoids



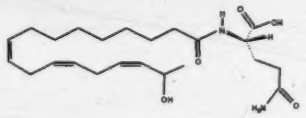
Herbivore elicitors

Bruchins from *Callosobruchus maculatus* oviposition fluid

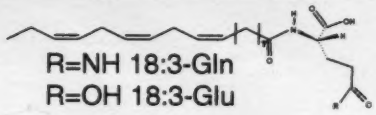


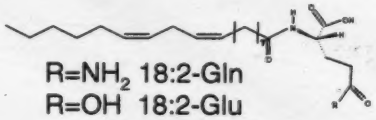


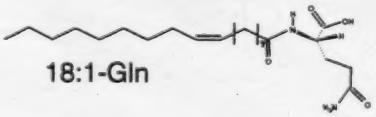
Volicitin from *Spodoptera exigua* oral secretion

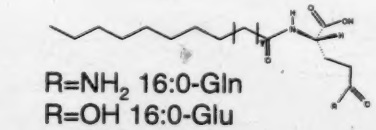


FACs from *Manduca spp.* oral secretion









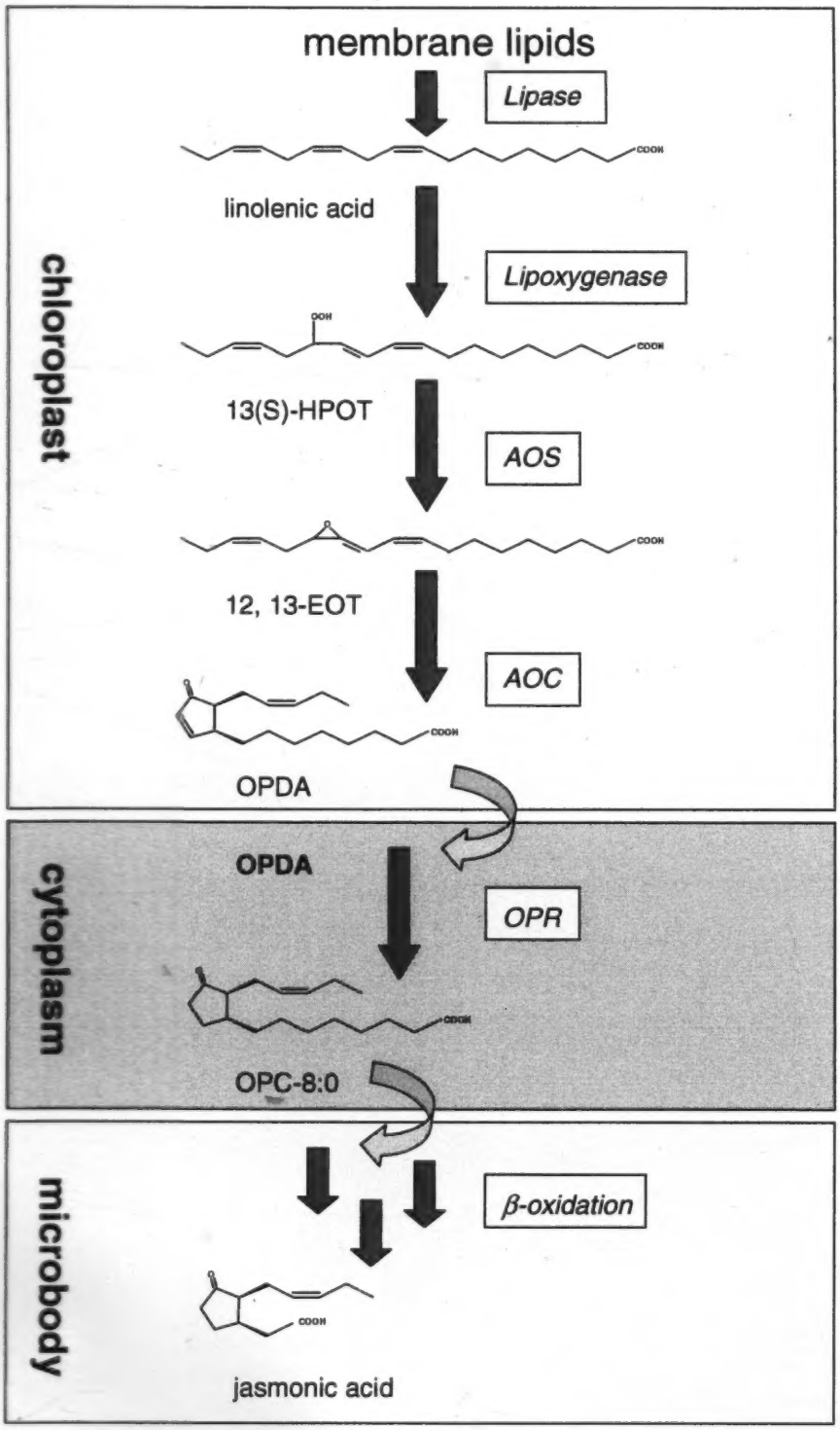
β-glucosidase from *Pieris brassicae* oral secretion

C-3 Wound Signal Transduction: the Octadecanoid Pathway

The octadecanoid pathway involves several intercellular and intracellular compartments (Figure 1-2) and results in the production of many compounds that are collectively known as jasmonates. The step catalyzed by allene oxide synthase (AOS) appears to be the rate-limiting step (Laudert and Weiler, 1998) and may serve a regulatory role in the pathway. The best characterized jasmonate, jasmonic acid (JA) and its methyl-ester (methyl-jasmonate, MeJa), are synthesized through this pathway from its alpha-linolenic acid (LA) precursor. Since the biosynthesis of OPDA (12-oxo-10,15(Z)-octadecatrienoic acid) occurs in the chloroplasts, the major sources of LA for JA biosynthesis may be monogalactosyl diacylglycerol and digalactosyl diacylglycerol (Simpson and Gardner, 1995). Galactosyl lipids are primarily located in chloroplasts and are partially degraded after wounding (Conconi *et al.*, 1996) or mechanical stimulation (Weiler *et al.*, 1993) and result in a rapid release of LA preceding the accumulation of JA (Conconi *et al.*, 1996; Mueller *et al.*, 1993). By analogy to mammalian eicosanoid biosynthesis, a phospholipase A (PLA) activity has been postulated to be involved in the release of LA during JA biosynthesis (Narvaez-Vasquez *et al.*, 1999). An increase of PLA activity could be detected in tobacco cells 2 h after elicitation (Roy *et al.*, 1995) and in soybean after treatment with extracts of a pathogenic fungus (Chandra *et al.*, 1996). However, evidence using transgenic antisense suggests that a phospholipase Dα is required for the wound-produced linoleic and linolenic acid (Zien *et al.*, 2001).

Figure 1-2: Biosynthesis of Jasmonic Acid

The biosynthesis of jasmonic acid takes place in three different cellular compartments. LA: -linolenic acid; LOX: lipoxygenase; AOS: allene oxide synthase; AOC: allene oxide cyclase; OPR: 12-oxo-phytodienoic acid reductase; 13(S)-HPOT: (9Z,11E, 15Z, 13S)-13-hydroperoxy-9,11,15-octadecatrienoic acid; 12,13-EOT: (9Z, 11E,15Z,13S,12R-12,13-epoxy-9,11,15-octadecatrienoic acid; OPDA: 12-oxo-10,15(Z)-octadecatrienoic acid; OPC-8:0: 3-oxo-2(2'(Z)-pentenyl)-cyclopentane-1-octanoic acid; JA, jasmonic acid. Adapted from Schaller (2001).


membrane lipids
Lipase
linolenic acid
Lipoxygenase
13(S)-HPOT
AOS
12, 13-EOT
AOC
OPDA
chloroplast
OPDA
OPR
OPC-8:0
cytoplasm
β-oxidation
jasmonic acid
microbody

C-4 Perception of JA and Induction of Responses

The JA signal is likely transduced by its binding to receptors. However, to date, no receptors have been identified. Three octadecanoids are known to induce defense responses in *Arabidopsis*: OPDA, jasmonic acid, and MeJa. OPDA and the jasmonates (JA and MeJa) are probably perceived by different receptors and may account for some of the specificity in the wound response. That these octadecanoids are perceived differently is evidenced by the fact that *VSP* transcription in *Arabidopsis* and stamen development are induced by jasmonic acid but not by OPDA (Ishiguro *et al.*, 2001; Stintzi *et al.*, 2001). It would be expected that membrane-spanning receptor proteins would be identified by mutants that are insensitive to octadecanoids; however, exhaustive mutant screens for insensitivity to MeJa and coronatine, a structural analogue to MeJa, have only identified alleles of *coi1* and jasmonate resistant (*jar1*) (Staswick *et al.*, 1992; Feys *et al.*, 1994). This may suggest that JA receptors are redundant, or that *COI1* and *JAR1* function in JA perception but, neither has homology to known receptor proteins. Regardless of how JA is perceived, the plant responds by upregulating a specific suite of genes.

C-5 Wound Signaling Pathways: Transcriptional Responses and Cross-talk

In addition to jasmonates, other plant hormones are also involved in defense responses. The specificity or generality of a stress response may be explained by which signal cascades are recruited and there is evidence that several distinct signaling pathways have components that overlap. Microarray analysis of 41 JA-responsive *Arabidopsis* genes reveals that three also respond to ethylene, auxin, and

salicylic acid (Sasaki *et al.*, 2001). Another microarray analysis of 2,375 selected *Arabidopsis* genes revealed that 169 mRNAs responded to multiple signal molecules including MeJa, SA, and ethylene (Schenk *et al.*, 2000). There are several examples of the requirement of ethylene for the induction of defense genes. For instance, defense genes such as *PDF1.2* in *Arabidopsis PI* in tomato and two PR genes *PR1b* and osmotin from tobacco all show maximal induction when JA and ethylene are both applied (Penninckx *et al.*, 1998; O'Donnell *et al.*, 1996; Xu *et al.*, 1994).

Plant responses become more difficult to interpret when damage occurs as a result of herbivory instead of mechanical or chemical induction. For example, phloem-feeding whiteflies can simultaneously induce both SA- and JA-regulated genes, such as those involved in lignin biosynthesis, SA biosynthesis, oxidative burst, and pathogenesis related and JA-responsive PR (pathogenesis related) genes (Walling, 2000). Nicotine accumulation in tobacco is found to be attenuated after feeding by (or the application of regurgitant from) the tobacco hornworm (*Manduca sexta*) when compared to mechanical wounding (Halitschke *et al.*, 2001; Schittko *et al.*, 2000). The attenuation is due to an ethylene burst that occurs after feeding which antagonizes the JA responsive expression of the nicotine biosynthetic genes, *NaPMT1* and *NaPMT2* (Winz and Baldwin, 2001; Kahl *et al.*, 2000; McCloud and Baldwin, 1997). Additionally, there are herbivore-induced genes that do not respond to known wound-signaling pathways. For instance, silverleaf whitefly feeding on squash induces *SLW3*, a gene that is not responsive to any other known wound signal (van de Ven *et al.*, 2000). Additionally, a wound-inducible tobacco peroxidase localized to the vascular system does not respond to known inducers of the wound response such

as jasmonate, 1-aminocycopropane-1-carboxylate, spermine, phytohormones or other stress treatments, suggesting that additional defense pathways await discovery (Sasaki *et al.*, 2002).

These examples reveal that the interactions of these pathways are complex in nature. Perhaps resolution of these problems will have to wait until they are approached using microarray technologies capable of surveying the expression of thousands of genes at a time. In fact this approach is already being used in *Arabidopsis* to analyze the interactions between wounding, pathogen, abiotic stress and hormonal responses (Cheong *et al.*, 2002). However, it should be noted that studies based on exogenous application of elicitors may suffer from unrealistic within-plant physiological concentrations or distributions of elicitors and the results should be interpreted with caution. Differences between chemically and biologically induced resistance have already been reported (Schweizer *et al.*, 1997; Molina *et al.*, 1999). Interpreting pathway interactions after herbivory is further complicated because it is also possible that specific insect-elicitors may also be altering the wound response.

C-6 Insect Elicitors and Insect Specific Plant Responses

Several insect-derived elicitors have been identified that provoke specific responses or defenses from plants which are clearly different from mechanical wounding. This suggests that the plant may respond to the specific pest. One recently identified type of insect-elicitor, called bruchins, was found in the oviposition fluid of weevils (Bruchidae), and was determined to induce a novel type of defense response

in peas. These elicitors are long chain diols that are mono- and diesterified with 3-hydroxypropanoic acid (Figure 1-1). In certain genotypes of peas, the bruchin elicites neoplastic growth on pods, which lifts the egg out of the oviposition site and may expose the larvae to predators, parasites, and desiccation (Doss *et al.*, 2000). Another type of insect-elicitor has been isolated from the oral secretions of lepidopteran larvae. These elicit indirect defense responses. One such orally-derived elicitor is the lytic enzyme ß-glucosidase, isolated from *Pieris brassicae*, which elicits the release of terpenoid volatiles from cabbage leaves (Mattiacci *et al.*, 1995). A second type of oral elicitors are fatty acid conjugates, FACs (Figure 1-1), are found in the regurgitant of larval Sphingidae (Halitschke *et al.*, 2001), Noctuidae, and Geometridae (Alborn *et al.*, 1997; Pohnert *et al.*, 1999). The FAC volicitin (N-17-hydroxylinolenoyl-L-glutamine) from *Spodoptera exigua*, induces excised maize seedlings to release the identical blend of volatile terpenoids and indole as when damaged by caterpillar feeding (Alborn *et al.*, 1997). The released volatile organic compounds (VOCs) can act as a scent beacon for predators and parasitoids. In maize, the blend of terpenoids and indole that are released after the application of volicitin are attractive to the generalist endoparasitic wasp *Cotesia marginiventris* (Turlings and Benrey, 1998). It appears that the alteration of VOC emission after herbivory may be common-place among plants. Interactions between plant-caterpillar-parasitoid (Dicke and van Loon, 2000), plant–leaf beetle–egg parasitoid (Meiners and Hilker, 2000), plant–spider mite–predatory mite, and plant–caterpillar–predatory bug interactions (Kessler and Baldwin, 2001) appear to be mediated by VOC emission.

In addition to changes in VOCs, other specific responses caused by insect herbivory include differences in gene expression. Compared to mechanical wounding, herbivory of potato (*Solanum tuberosum*) by tobacco hornworm results in a more rapid induction of proteinase inhibitor II and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Korth and Dixon, 1997). The application of hornworm regurgitant fed to leaves through the petiole also induces transcript accumulation and shifts ahead the time of transcript accumulation parallel to those seen by herbivory (Korth and Dixon, 1997). It is also likely that insects modify plant defense responses to their own advantage. In *Arabidopsis*, feeding by the larvae of cabbage butterfly (*Pieris rapae*) also results in different transcription profiles than those seen after mechanical wounding (Reymond *et al.*, 2000). The major difference is the relative lack of water stress-induced expression during insect feeding compared to mechanical wounding. By contrast, a hevein-like protein (*HEL*), is specifically induced by *P. rapae* but not at all by mechanical wounding. This suggests that the plants are responding to an insect specific cue. More recently, several genes in tobacco (*Nicotiana attenuata*) have been demonstrated to respond differentially to tobacco hornworm herbivory (Schittko *et al.*, 2001). In this system, the fatty acid-amino acid conjugates in herbivore oral secretions were shown to be responsible for the differences (Halitschke *et al.*, 2001). These examples from other insect-plant interactions suggests that woody plants may react in a similar manner. It would be surprising if specific plant-insect interactions did not exist in the *Populus* model system, and this is in part the rationale for this thesis.

C-7 Overview of Induced Defense Proteins in Plants

The induction of plant defenses by wounding and herbivory involves the rapid accumulation of proteins and phytochemicals that have the purposes of deterring feeding, poisoning, or starving insect herbivores feeding on the plant. There is a substantial diversity in wound-induced defense proteins which include proteinase inhibitors, oxidative enzymes, chitinases, and enzymes involved in the production of toxic secondary metabolites such as phenylpropanoids (Constabel, 1999). More is known about genes coding for defensive proteins than genes involved in phytochemical production. This may be because single genes coding for proteins with potential anti-insect properties have been preferred for transgenic analysis. However, it may soon be common to analyze pathways involved in the production of complex phytochemicals by transforming entire metabolic paths into test plant systems.

Prominent among the wound-induced proteins are proteinase inhibitors (PIs). PIs are separated into different classes: inhibitors of serine, cysteine, aspartic, or metallo-proteases (Ryan, 1990). Classes of PIs are further divided into families based on amino-acid sequence similarity. Of the eight distinct known families of serine protease inhibitors (Ryan, 1990; Richardson, 1991), the wound- and herbivore-induced examples belong to the potato proteinase inhibitor I and II families, Bowman-Birk, and Kunitz-type families. PIs are effective against insects by reducing the availability of amino acids. Insects may compensate by increasing the amounts of trypsin secreted resulting in a net loss of essential sulfur containing amino-acids with lethal results (Ryan, 1990; Duffey and Stout, 1996). The anti-herbivore potential of tomato inhibitor II overexpressed in tobacco was clearly demonstrated against

tobacco hornworm (Johnson *et al.*, 1989). Bowman-Birk PIs are often found in seeds of legumes and cereals, and have only been shown to be wound-induced in alfalfa (Brown *et al.*, 1985). However, the effectiveness of a cowpea PI in protecting transgenic tobacco against *Heliothis virescens* affirmed the defensive function of the Bowman-Birk family of PIs (Hilder *et al.*, 1987). The Kunitz-type PIs are found in legumes and cereals, although poplar and aspen have wound-inducible homologs (Bradshaw *et al.*, 1989; Haruta *et al.*, 2001). The aspen PI has the ability to inhibit bovine trypsin in vitro (Haruta *et al.*, 2001), demonstrating that active woody plant defense is similar to herbaceous species. Cysteine PIs and cystatins are found in the seeds of legumes and cereals (Richardson, 1991). In addition, cystatin genes are wound-induced in potato (Hildmann *et al.*, 1992, Bergey *et al.*, 1996) and potato plants overexpressing cystatin demonstrate inhibitory activity against coleopteran proteases (Benchekroun *et al.*, 1995). Although several PIs have demonstrated effectiveness against specific insects it should be noted that PIs are specific for their specific protease targets. Distinct proteases within an insect may have different susceptibilities to specific PIs, and some insects actively alter their profile of digestive proteases in response to PIs (Jongsma *et al.*, 1995; Broadway, 1996).

Plants also have several inducible, stress related oxidative enzymes that have the potential of playing a role in antinutritive defense. The anti-nutritive effects of oxidative enzymes depend on their ability to destroy essential nutrients in the ingested plant matter. Factors such as protein quantity and quality, redox potential in the gut, and the ability of insects to generate anti-oxidants all influence the effectiveness of oxidative enzymes in defense (Duffey and Stout, 1996).

Polyphenol oxidase (PPO) is a common enzyme that is often responsible for the browning of damaged plant tissues. PPOs have a copper based active site and use molecular oxygen to oxidize phenolic substrates such as caffeic acid. During insect feeding, the resulting breakdown of cellular compartalization allows PPO to mix with its substrates to produce highly reactive *ortho*-quinones which spontaneously polymerize other biomolecules. The alkylation of amine and sulfhydryl groups in proteins results in protein modification, cross-linking and even precipitation, reducing the availability and assimilation of amino acids by the insect (Felton *et al.*, 1992; Duffey and Stout, 1996). The results of PPO activity can negatively impact the growth and performance of insects; the growth of tomato fruit worm (*Helicoverpa zea*) and beet armyworm (*Spodoptera exigua*) varies inversely with the quantity of PPO present in tomato tissue (Felton *et al.*, 1989; Stout *et al.*, 1996). Other oxidative enzymes with similar modes of action to PPO are peroxidases, heme-containing enzymes that use H_2O_2 to oxidize phenolics, ascorbate and indole acetic acid (Butt, 1980). Like PPO, peroxidases are capable of oxidizing a variety of phenolics resulting in the alkylation of dietary proteins. However, there is little evidence for adequate H_2O_2 levels in wounded plant tissues to be effective. More likely, the primary role of peroxidases is in the lignification and reinforcement of cell walls. Following damage by the forest tent caterpillar (*Malacosoma disstria* (FTC), leaf toughness in poplar increased both locally and systemically (Robison and Raffa, 1994).

Lipoxygenases may play two different roles in defense. As previously mentioned, lipoxygenase is required for the synthesis of octadecanoids. In addition, it may have a direct role in the antinutritive defense by destroying polyunsaturated fatty

acids (insect nutrients) and by generating free radicals that alkylate dietary amino acids (Duffey and Stout, 1996; Duffey and Felton, 1991). Ascorbate oxidase, another oxidative enzyme, does not act directly in the antinutritive defense but prevents ascorbic acid from acting as an antioxidant and free radical scavenger. Therefore, the presence of ascorbate oxidase would enhance the effectiveness of the other aforementioned oxidases. Recently, it has been demonstrated that an ascorbate recycling system in the midgut lumen can act as an effective antioxidant defense in caterpillars that feed on prooxidant-rich foods (Barbehenn *et al.*, 2001).

Another commonly wound-induced group of proteins are chitin-binding proteins and chitinases. Chitinases may be effective against insect herbivores by weakening the gut lining, making them more susceptible to phytotoxins or pathogens. Although chitinases have been studied extensively as defenses against pathogens and have demonstrated *in vitro* and *in vivo* antifungal activity, antiherbivore activity has not been reported. However, a tobacco hornworm chitinase overexpressed in tobacco was effective in reducing the growth of *Heliothis virescens* (Ding *et al.*, 1998). It is also possible that the main role of chitinases is in defense against opportunistic fungal pathogens taking advantage of insect inflicted wounds.

D. POPULUS WOUND-INDUCTION AND DEFENSE

D-1 Insect Herbivore Defense in *Populus*

Relatively little is known about *Populus* defense, even though *Populus spp.* are commonly defoliated by the larvae of Lepidopteron species such as FTC, gypsy moth (*Lymantria dispar*), and large aspen tortrix (*Choristoneura conflictana*). The

knowledge gained to date has revealed that *Populus spp.* share many defense responses with herbaceous species such as potato (Table 1-1.). These include the activation of proteinaceous defenses such as PIs (Bradshaw *et al.*, 1989; Hollick and Gordon, 1995; Haruta *et al.*, 2001) and chitinases (Davis *et al.*, 1991), the accumulation of condensed tannins (Peters and Constabel, 2002) as well as the activation of oxidative enzymes such as the inducible gene PPO (Clausen *et al.*, 1989; Constabel *et al.*, 2000). The induction of many of these by the exogenous application of the wound-signaling intermediate methyl-jasmonate (MeJa) revealed that the octadecanoid pathway is involved in the wound- induction of defenses in *Populus* as it is in herbaceous plants. As in tomato, poplar trypsin inhibitor (TI) and PPO are induced systemically in upper unwounded leaves when lower leaves are damaged by artificial or herbivore damage (Hollick and Gordon, 1995; Constabel *et al.*, 2000; Haruta *et al.*, 2001) suggesting that a mobile systemic signal is involved.

D-2 Protein Based Induced Defenses in *Populus*

As in tomato and potato, PIs make up a significant portion of the proteinaceous defenses in *Populus* spp. There at least three different families of Kunitz serine PIs, and each demonstrates a different wound-induction profile (Mary Christopher and Peter Constabel, unpublished data). One shows amino acid similarity to a TI from *Theobroma*

cacao (45% identity) and the second has similarity to a TI from *Nicotiana tabacum* (31% identity) (Mary Christopher and Peter Constabel, unpublished data). The remaining family is a multigene family containing the *win3* (*w*ound-*in*ducible)

Table 1-1: Wound-induced Proteins in Potato and *Populus spp.*

A comparison of wound-induced proteins and predicted proteins in the woody perennials *Populus spp.* and the herbaceous annual potato (*Solanum tuberosum*). NA designates proteins without cloned homologs or expression data.

Wound-induced proteins in *Populus spp.* (accession number)	***Populus* references**	**Potato (*Solanum tuberosum*)** (accession number)	**Potato References**
Vegetative storage proteins (Vsp)			
Bark storage protein (BSPA) (Q07469), (CAA49669), Vsp-win4 (AAA16342), VSP-PNI288 (AAK01124)	;Davis et al., 1993; Coleman and Chen 1993; Lawrence et al., 2001	N/A	N/A
Chitinases			
win6 (AAA96702), win8 (P16061)	Davis et al, 1991	ChtA1 (AAB96340), ChtA2 (AAB96341),ChtA3 (AAB81962), ChtA4 (AAB81963), ChtB1 (AAA18332), ChtB2 (AAA17408), ChtB3 (AAA17409), ChtB4 (AAA17410), ChtC1 (AAC24807), ChtC2 (AAC24808)	Buchter et al., 1997; Beerhues and Kombrink, 1994; Ancillio et al., 1999
Trypsin inhibitors			
gwin3 (CAA33539), TI-1 (AF349441), TI-2 (AF349442), TI-3 (AF349443), win3.12 (AAA02863)	Bradshaw et al, 1990; Hollick and Gordon, 1995; Haruta et al., 2001	PIA (T07411), PID (T07413), PIE (T07414)	Ishikawa et al., 1994
Oxidative enzymes			
polytphenol oxidase (PPO) (AAG21983), (AAK53414)	Constabel et al., 2000: Haruta et al, 2001	PPO (AAA85121), PPO (AAA85122), PPO (AAA85123)	Thygesen, Dry and Robinson, 1995
Phenylpropanoid path and related enzymes			
dihydroflavonol 4-reductase (DFR)	Peters and Constabel, unpublished	NA	NA
phenylalanine ammonia-lyase (PAL)	Peters and Constabel, unpublished	PAL-1 (X63103), PAL-2 (X63104)	Joos and Hahlbrock, 1992
4-coumarateCoA ligase (4CL)	Peters and Constabel, unpublished	St4C1-1 (AAA33842), 4CL-2a (AF150686), 4CL-2b (AF150687)	Becker-Andre, Schulze-Lefert and Hahlbrock, 1991
chalcone synthase (CHS)	Peters and Constabel, unpublished	CHS 1a (AAB67734), CHS 1b (AAB67735), naringenin-CHS 2 (JC5136)	Jeon et al., 1996

Wound-induced ESTs: presumed enzymatic activities			
1-aminocyclopropane-1-carboxylic acid (ACC) oxidase	Christopher, Yip and Constabel, unpublished	ACC oxidase (AAK68075), ACC oxidase (AAK68076)	NA
AP-2 domain transcription factor	Christopher, Yip and Constabel, unpublished	NA	NA
AWI31-like	Christopher, Yip and Constabel, unpublished	NA	NA
hydroperoxide lyase (HPL)	Christopher, Yip and Constabel, unpublished	fatty acid hydroperoxide lyase (CAC44040)	Vancanneyt et al., 2001
isoflavanone reductase	Christopher, Yip and Constabel, unpublished	NA	NA
lipoxygenase	Christopher, Yip and Constabel, unpublished	Lox1 (CAA64765), Lox2 (CAA64766), Lox3 (CAA64764),check status of lox1-3? LOX (AAB31252)	Royo et al., 1996; Geerts, Feltkamp and Rosahl, 1994
P450-like	Christopher, Yip and Constabel, unpublished	NA	NA
Secoisolariciresinol dehydrogenase	Christopher, Yip and Constabel, unpublished	NA	NA

homologs. *Win3* shows amino acid similarity to sporamins and Kunitz-type TIs from legume seeds and responds to wounding both locally and systemically (Bradshaw *et al.*, 1989; Hollick and Gordon, 1993). In aspen, the TI family contains at least three members and is induced at the mRNA and protein level locally and systemically by wounding and is also induced by MeJa demonstrating the involvement of the octadecanoid path (Haruta *et al.*, 2001). The product of one of the aspen *win 3* homologs was shown to encode a functional TI; *PtTI2* has been over-expressed in *Escherichia coli* and demonstrates inhibition of bovine trypsin *in vitro*.

In addition to TIs, wounding in poplar results in the expression of chitinases that are induced both locally and systemically (Clarke *et al.*, 1994; Davis *et al.*, 1991). Poplar trees have at least two different chitinase genes, *win6* and *win8* that belong to multigene families (Davis *et al.*, 1991). Both predicted proteins are expected to be highly acidic and likely accumulate in the vacuole. There are also vegetative storage proteins (VSPs) of unknown function which are wound-inducible and which may also play a part of the proteinaceous defense (see section 3.4).

D-3 Secondary Metabolism (Phytochemical) Defense in *Populus*

Although proteinaceous defenses make up a significant portion of the arsenal available against insect herbivores, *Populus spp* are also armed with phytochemical defenses. Preliminary evidence from a survey of interspecific poplar hybrids suggest that the performance of lepidopteran defoliators varied in relation to differing levels of defensive chemicals (Robison and Raffa, 1994). The defensive chemistry of aspen is comprised almost entirely of phenolic compounds, in particular tannins and

phenolic glycosides (Palo, 1984). Condensed tannins, consisting of proanthocyanidins (flavonoid polymers that are able to strongly bind macromolecules through hydrogen, hydrophobic, ionic and covalent bonds), are found at concentrations of up to 18% dry weight in aspen leaves (Lindroth and Hwang, 1996). Tannins have antinutritive properties and are effective in defense by reducing the palatability of leaves. Tannins extracted from aspen leaves are effective at precipitating protein (Peters and Constabel, 2002). This can result in an effective decrease in the food quality of ingested leaves leading to decreased growth of the large aspen tortrix (*Choristoneura conflictana*) (Bryant *et al.* 1987). Several genes encoding enzymes in the phenylpropanoid pathway involved in tannin synthesis in aspen have recently been cloned and their expression analyzed (Peters and Constabel, 2002). Mechanical wounding and herbivory by both satin moth and FTC resulted in the induction of dihydroflavonol reductase (DFR), leucoanthocyanidin reductase (LAR), phenylalanine ammonia-lyase (PAL), 4-coumarateCoA ligase (4CL), chalcone synthase (CHS), and anthocyanidin synthase (ANS). These phenylpropanoid genes all respond to a systemic signal, as evidenced by their induction in upper unwounded leaves. This signal is likely transduced through the octadecanoid path, since DFR is induced by the application of MeJa.

Phenolic glycosides identified in aspen include salicin, tremuloidin, salicortin, and tremulacin and the toxic effects of these phytochemicals on gypsy moth and forest tent caterpillar have been documented (Lindroth, 1991; Lindroth *et al.*, 1987). Herbivory by the large aspen tortix induces the accumulation of two toxic phenolic glycosides, salicortin and tremulacin, within 24 hours (Clausen *et al.*, 1989).

Salicortin is converted to 6-Hydroxy-2-cyclohexenone (6-HCH) and salicin upon mechanical damage to the leaf; a reaction that appears to be enzymatic (Clausen *et al.*, 1989). Although 6-HCH is itself toxic, it may be converted to catechol under the basic conditions found in the guts of many forest lepidopteron defoliators (Haruta *et al.*, 2001). Catechol is both toxic to insects and can be activated by the wound-inducible enzyme PPO (Constabel *et al.*, 2000). Furthermore, PPO itself is induced in poplar and aspen. Following mechanical wounding and MeJa treatment, PPO mRNA transcripts are detectable within 8 h and peak around 16 and 24 h in wounded and unwounded leaves respectively followed by measurable increases in PPO enzyme activity (Constabel *et al.*, 2000; Haruta *et al.*, 2001). PPO activity increases in wounded and unwound leaves after 72 h respectively.

As summarized above, poplar and aspen both use chemical and proteinaceous defenses. However, these strategies appear to be emphasized differently. For example, hybrid poplar appear to rely less on the accumulation of secondary metabolites such as phenolic glycosides, tannins and phenylpropanoids and rely more on proteinaceous defenses such as TIs. DFR is much more strongly induced in aspen than in poplar (Peters and Constabel, 2002). The converse is true with TI protein, which is more strongly induced in poplar leaves than they are in aspen leaves (Ian Major and Peter Constabel, unpublished data). It is noted that the defenses known to date are likely only a subset of those used by *Populus*. It is likely that the emphasis on individual components of the defense by different *Populus* species may reflect the synergistic efficacy of such defense combinations. The natural use of different defense combinations may yield insight into how they function in defense and

ultimately how to create transgene defense pyramids (the use of multiple defense transgenes) in crops.

D-4 Vegetative storage proteins and induction during wound response

An intriguing feature of the poplar defense response is the accumulation of a set of proteins of unknown biochemical function, which are very similar in sequence to a vegetative storage proteins previously identified (bark storage protein (BSP) and VSP-*win4.5*) (Davis *et al.*, 1993). Vegetative storage proteins (VSPs) accumulate in specific cell types or tissues during plant development and appear to serve as temporary nitrogen reserves. VSPs most often accumulate in vacuoles during periods of high carbon and nitrogen availability and are later degraded during other stages of development. The term VSP is a functional designation and contains members without sequence or structural similarity. LOX, patatin, and acid phosphatase were previously designated as VSPs by their expression patterns and were later determined to have enzymatic activity (Tranbarger *et al.*, 1991; Andrews *et al.*, 1988; DeWald *et al.*, 1992). Even protease inhibitors were considered vacuolar reserves (Shumway *et al.*, 1970) until their role in defense was discovered (Green and Ryan, 1972). The accumulation of proteinaceous defences for storage in seeds, tubers, bark and other storage tissues appears to be a strategy designed to protect the resources invested. The expression of defenses in vulnerable developing tissues or sinks likely reflects a similar strategy.

In general, VSPs in *Populus* display expression patterns linked to nutrient-status and development (Davis *et al.*, 1993), much like VSPs in herbaceous plants.

However, in addition to responding to nitrogen status, BSP and VSP-*win4.5* are induced systemically in the shoot apex (Davis *et al.*, 1993) and VSP-*win4.5* is induced both locally and systemically in leaves. A similar interaction was previously identified in soya, where the nitrogen supply-regulated VSPα and VSPβ were found to be induced by wounding, the wound signaling intermediates MeJa and Jasmonic acid, and water deficit (Franceschi and Grimes, 1991; Mason and Mullet, 1990; Mason *et. al.*, 1992; Staswick *et al.*, 1991). These VSPs were later found to have acid phosphatase activity (DeWald *et al.*, 1992). Soybean plants can accumulate VSPs to more than 45% of the soluble leaf protein (Mason and Mullet, 1990). In *Arabidopsis*, a VSPα homolog, *Atvsp*, has been cloned and its expression responds to many of the same stimuli as VSPα (Berger *et al.*, 1995). Like soybean AP, *Atvsp* mRNA is induced locally and systemically. The conserved wound-induction of VSPs in diverse plants suggests that they may perform an important function during the wound response, although this function still remains to be defined.

E. MOLECULAR TECHNIQUES AND APPROACHES

E-1 Molecular Techniques Used to Study Plant Transcriptional Changes

The rapid wound and herbivore induction of genes important for plant defense against herbivores is a key feature of higher plant defense. If their changes in gene expression are adaptive, then novel defense genes and mechanisms can be discovered by searching for wound-induced genes. Screening for differentially expressed genes is thus an important strategy for studies of plant defense. In the last 10 years, differential screening technology has evolved rapidly and currently high-throughput

tools for genome-wide transcript profiling, such as expressed sequence tags (ESTs) and microarray analysis, are becoming widely available. In traditional screening methods such as differential hybridization, the hybridization pattern of the total content of a cDNA library is compared between two samples (Maniatis *et al.*, 1982). However, the most abundant transcripts are also displayed, leading to strong signals from non-relevant genes and thus a very low labor efficiency. This problem has been solved partly by normalization and subtraction (Hedrick, 1984) even then, many interesting low-abundance differentially expressed genes are missed because of the low amplification of the hybridization signal (Wang and Brown, 1991). Presently, the common procedures for the analysis of differential expression includes macro- and microarrays, subtractive libraries, AFLP-cDNA display (amplified fragment length polymorphism – complementary DNA display), and DD-RT (differential display-reverse transcription). Each method has its own advantages and disadvantages.

E-2 AFLP-cDNA

AFLP-cDNA (amplified fragment length polymorphism-cDNA) is based on the ability to detect polymorphisms (differences caused by mutations i.e. different alleles) in mRNA transcripts as changes in restriction sites (Savelkoul *et al.*, 1999). The patterns obtained by different strains (resistant or succeptible) are polymorphic due to mutations in the restriction sites, mutations that alter the annealing of primers used in PCR extension, and/or insertion and deletion events in the cDNA. In addition, tissue samples reflecting different treatments may express subsets of genes differentially leading to additional visualized bands. It is this latter set of bands that

are useful for comparative gene expression. Different patterns from the same sample sets can be generated by extending the length of primers. An extension of one selective nucleotide in the primer amplifies 1 of 4 of the ligated fragments, whereas three selective nucleotides in both primers amplify 1 of 4,096 of the fragments (Savelkoul *et al.*, 1999).

This technique has recently been used to identify early induced genes in a fungal pathogen-plant interaction between tomato and *Cladosporium fulvum* (Durrant *et al.*, 2000). Of the estimated 30,000 fragments inspected, 290 showed altered expression. Thirteen cDNA clones were expressed within 4 h after Avr9 was added. Few of the reported fragments were over 200 bp making it necessary to identify longer ESTs for Northern analysis.

E-3 DD-RT

Like AFLP-cDNA, the basic principle of DD-RT is that cDNAs, representing fragments of mRNAs, will be amplified by PCR, and those cDNAs that are differentially expressed between treatments will be revealed as novel bands in some but not other samples appearing on the polyacrylamide gel. The principle of the technique is to PCR amplify subpopulations of cDNA using random primer combinations to produce random populations of PCR products of different lengths (Liang and Pardee, 1992). Original statistics indicated that 80 primer combinations would be sufficient to cover the whole transcript mass (Liang *et al.*, 1993). By carrying out the amplifications in both control and treated tissue, transcripts more highly expressed in one of the tissues can be identified. The method is rapid,

producing band patterns in two days and more than two samples can be compared. However, the subsequent analysis of the differential display products reveals a major drawback: the frequency of false positives may be as high as 50–75% of the excised bands (Wan *et al.*, 1996; Debouck, 1995; Liang *et al.*, 1993). In addition, the cDNA fragments obtained from differential display are short (typically 100–500 bp) and correspond to the 3' end of the gene that represents mainly the 3' untranslated region and little of the coding region (Lievens *et al.*, 2001). Unless a model system is studied for which a significant amount of sequence information is available in public databases, labor-intensive full-length cDNA screening is needed to provide significant sequence homology, informative for gene classification and prediction of function.

As an example, DD-RT was employed in order to study molecular interactions that occur between rice (*Oryza sativa*) and rice blast fungus (*Magnaporthe grisea*) upon infection (Kim *et al.*, 2000). Approximately 800 cDNA fragments were apparently upregulated differentially and ranged in size from 100-350 bp. After screening a cDNA library for longer ESTs, 16 cDNA clones demonstrated the accumulation of related mRNA transcripts after fungal elicitor treatment. Thus, only 16 out of 800 fragments demonstrated induction using Northern analysis revealing the potential for isolating false positives using this technique.

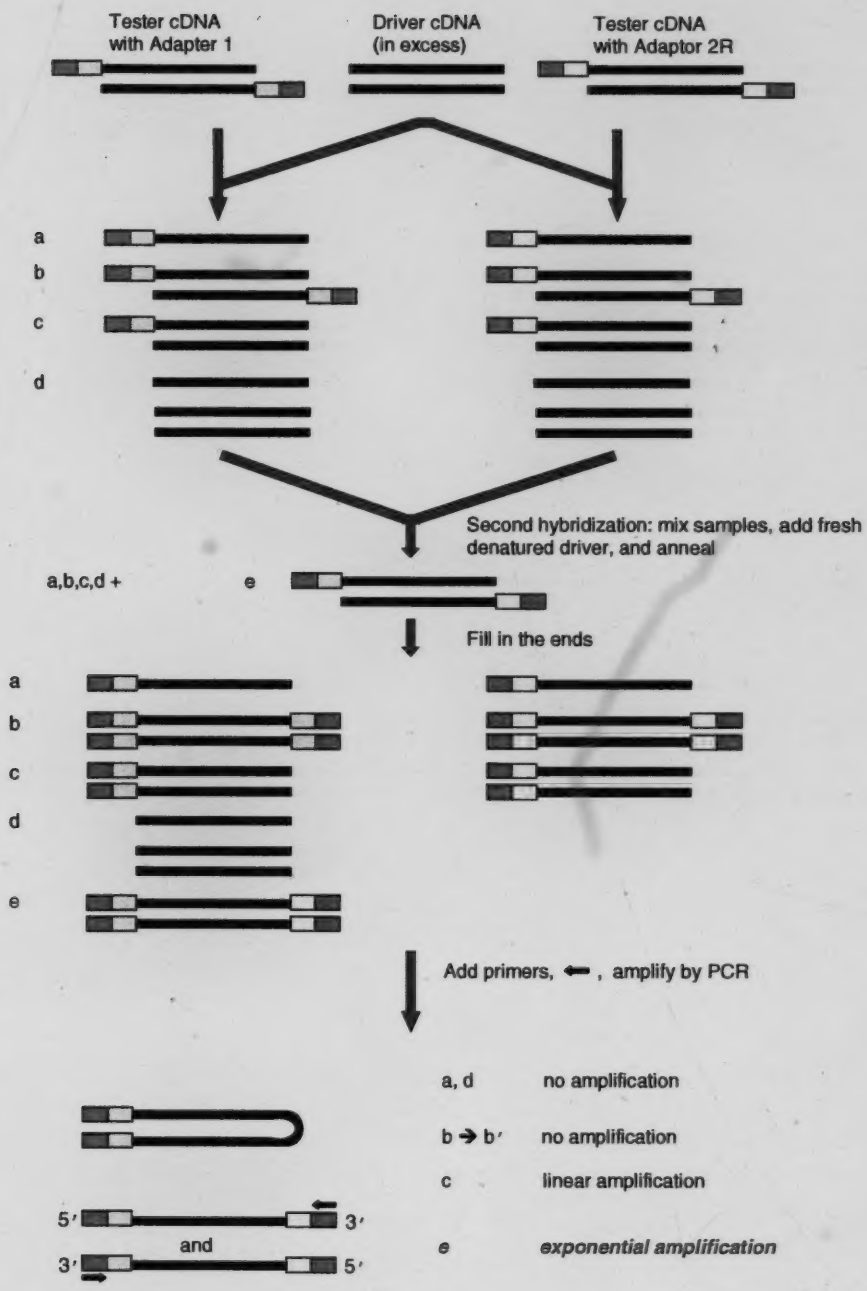
E-4 SSH

An extremely powerful method for isolating differential expressed genes is suppression subtractive hybridization (SSH) (Diatchenko *et al.*, 1996; Gurskaya *et al.*,

1996). SSH is a highly efficient and widely used PCR-based method for identifying differentially expressed genes (Yamagishi, 1999; von Stain *et al.*,1997; Yokomizo *et al.*, 1997). The basic principle is that two samples representing the control and treated tissues are compared at the molecular level resulting in the removal of common cDNA sequences and the enrichment of upregulated sequences from the treated sample. This is achieved by ligating adaptors to allow PCR amplification of upregulated cDNAs and removal of common cDNAs (normalization) by hybridization of the treated pool of cDNAs to the control pool. A key feature of the SSH method is simultaneous subtraction and normalization that makes it possible to equalize the abundance of target cDNAs in the subtracted population. As a result, rare differentially expressed transcripts can be enriched by ~1000-fold. The SSH technique is based on the suppression PCR effect that is mediated by long inverted terminal repeats attached to the ends of DNA fragments (Siebert, 1995). The SSH method normalizes sequence abundance within the amplified cDNA population and prevents amplification of undesirable DNA fragments by incorporating this suppression effect in a PCR amplification scheme (Figure 1-3). The SSH scheme includes the following main steps. First, the tester cDNA is subdivided into two samples and these samples are ligated with two different suppression adapters. Secondly, the tester cDNA (manipulated) is hybridized with excess driver (control). Finally, the tester cDNA molecules that are flanked only with different suppression adapters are amplified. It is this fraction which contains the enriched and normalized target cDNA (Diatchenko, 1999; Chenchik *et al.*, 1998; Diatchenko *et al.*, 1996;

Figure 1-3: Suppressive Subtractive Hybridization

Scheme for the suppressive subtractive hybridization (SSH) method adapted from (Diatchenko *et al.*, 1996). Solid lines represent the RsaI digested tester (manipulated) or driver (control) cDNA. Blue boxes represent the outer part of the adapter 1 longer strand and corresponding PCR primer P1 sequence. Green boxes represent the outer part of adapter 2R longer strand and corresponding PCR primer P2 sequence. Yellow boxes represent the inner part of the adapters and corresponding nested PCR primers PN1 and PN2. Although there is a primer binding sequence on both ends of type *e* molecules, the shorter overall homology at the two ends practically negates the suppression PCR effect – except for very short molecules.


Tester cDNA with Adapter 1
Driver cDNA (in excess)
Tester cDNA with Adaptor 2R
a
b
c
d
Second hybridization: mix samples, add fresh denatured driver, and anneal
a,b,c,d +
e
Fill in the ends
a
b
c
d
e
Add primers, ⬅ , amplify by PCR
a, d
no amplification
b → b'
no amplification
c
linear amplification
e
exponential amplification
5'
3'
and
3'
5'

Gurskaya *et al.*, 1996). Typically, the complexity of typical SSH samples is no more than 103 independent cDNA species (Rebrikov *et al.*, 2000).

In a project conducted to search for immediate early defense related genes, again involving rice and rice blast fungus, the SSH approach was used. A combination of cycloheximide treatments were used to suppress *de novo* protein synthesis, plus JA or benzothiadiazole (BTH) to induce a transcriptional response was used on rice seedlings (Xiong *et al.*, 2001). The initial screening of 768 random clones resulted in the identification of 34 distinct immediate early genes, 200-600bp, by JA, BTH and/or blast infection. The results of the sequence analysis revealed that 19 genes are homologous to *Arabidopsis* genes of unknown function or show no homology with sequences in the databases. Another project using SSH to identify mercury-induced gene expression in *Arabidopsis* allowed 31 induced clones out of the initial 576 randomly picked clones to be identified (Heidenreich *et al.*, 2001). These results suggest that the SSH technique is comparable or even slightly better than the DD-RT technique as nearly twice as many differentially genes were isolated.

The SSH has other advantages over the DD-RT technique. First, the analysis of complex banding patterns of cDNAs on polyacrylamide gels is not required. Second, the SSH method involves the cloning of the cDNA pool containing the differentially expressed genes prior to any analysis. This means that the cloned inserts can be sequenced, used for array analysis and used as template for radio-labeled random primed probes for Northern analysis. AFLP-cDNA and DD-RT involves the cloning of cDNAs after the screening which can be problematic since contamination of similar sized undesirable cDNAs is quite common.

E-5 Expression Analysis Using Arrays

In order to identify the actual differentially expressed genes in a subtractive library, large scale screening of the individual clones is still required. Arrays are an effective way to screen the large numbers of cDNAs produced by DDRT-PCR and SSH for differential expression. Many different approaches have been proposed to meet the necessity of large-scale screening of candidate cDNA fragments with low amounts of RNA. These include techniques such as slot blots (Vogeli-Lange, 1996) or dot blots (Mu *et al.*, 1994) and reverse-Northerns (Consalez, 1996), in combination with amplified RNA as a probe (Poirier, 1997). Arrays containing hundreds and even thousands of DNAs can be constructed to meet the necessity of larger scale screening. Dot blots and more densely packed macroarrays are spotted on nylon membranes by hand, with the aid of a dot blot grid apparatus, or by robotic spotters. Microarrays are a more advanced form of the macroarray and are comprised of DNA spotted on glass slides in a high density grid arrangement (Schena *et al.*, 1995). The arrays are used for a multiplex reaction—much like a large scale dot blot, where the microarrays serve as hybridization targets for cDNA transcribed from RNA extracted from tissue or cell lysates. Closely related genes and gene families may cross-hybridize. Heller *et al.* found cross hybridization between genes with 70–90% sequence homology and also between genes with short regions of identity over the length of the target (Heller *et al.*, 1997). The ability to obtain qualitative results from arrays is thus dependent on the specificity of the system. A comparison of microarray and macroarrays with Northerns demonstrates that the array data tends to be "compressed" – that is, it contains a lower dynamic range (Schena *et al.*, 1996). Although replication of

experiments will eliminate many false positives, as with any technique, confirmation of expression results from array analysis should be confirmed with an independent method such as RNase protection, RT-PCR, or northern analysis.

E-6 Identification of Gene Function by Database Comparison and Bioinformatic Analysis

The first step towards understanding the functions of newly identified genes is a similarity search for homologous sequences in databases. Similarity to genes of known function or to characterized domains of protein families gives insight to possible function. Database searches using variations of programs such as BLAST (basic local alignment search tool) allow efficient identification of the most similar sequences in the database. Whole genomes and millions of randomly selected ESTs have been generated and deposited in public and private databases. These can be used to go *in silico* from a partial cDNA to matching longer ESTs, if ESTs from the same species are available. The longer ESTs are more likely to be useful for classifying the gene product. The Constabel lab has initiated EST projects by sequencing a wound-induced library (Mary Christopher and Lynn Yip, unpublished) and has independently sequenced clones from the SSH library created by this author (Mary Christopher and Joe Patton, unpublished). These "in house" sequences are invaluable for confirming and extending cDNA sequences generated by related projects such as this one. The *Populus* model system is also accumulating EST data in public accessible databases. For example, the PopulusDB website (http://poppel.fysbot.umu.se/) allows users to use BLAST homology searches against

ESTs and their related predicted protein sequences from hybrid aspen (*Populus tremula x P. tremuloides*). Furthermore, *Populus* EST sequences can also be searched through the NCBI website (http://www.ncbi.nlm.nih.gov/). There is sufficient *Populus* EST data available to complement molecular techniques such as SSH and will facilitate the identification of differentially expressed genes and the prediction of their function in insect-herbivore defense in poplar.

F. THESIS JUSTIFICATION AND OBJECTIVES

F-1 Project Justification

As a woody model system, *Populus* has many advantages. It is known that poplar have wound-induced defenses (Robison and Raffa, 1994) and accumulate defense related proteins such as PPO and TI after mechanical damage, FTC herbivory and MeJa application (Constabel *et al.*, 2000). The transcriptional induction of wound-related genes after MeJa application suggests that *Populus* uses the octadecanoid pathway to mediate wound-induction. As in tomato, the systemic signal moves from wounded leaves to unwounded leaves similar to sucrose transport (Davis *et al.*, 1991). While proteinaceous defenses such as TI are common among diverse plants there are great differences between plant species in their use of secondary metabolites, and likely other defenses. In poplar, the only enzymes involved in the production of secondary metabolites that have been cloned are involved in tannin synthesis through the phenylpropanoid pathway. The enzymes involved in making phenolic glycosides are presently unknown. It is likely that other pathways involved in making defensive secondary compounds in *Populus* species remain to be

discovered, for example volatiles which could attract parasitoids and predators. Furthermore, recent microarray experiments using *Arabidopsis* indicate that hundreds of genes respond to wounding (Cheong *et al.*, 2002) suggesting there are many more inducible genes to be discovered in poplar as well.

In poplar, recent molecular techniques such as SSH have not been used to identify herbivore-induced genes. Since poplar pests such as FTC are seasonally available it is possible to use the poplar model system to analyze changes in the transcript profile of a woody perennial plant after insect herbivory. This is one of the first such studies performed on a woody perennial or forest tree.

F-2 Objective of this Thesis

The objective of this thesis is to test the hypothesis that FTC herbivory and mechanical wounding systemically induce defense genes in poplar leaves. This will be accomplished by the differential screening of macroarrays spotted with a poplar leaf SSH cDNA library. The library will be created using FTC systemically-induced leaves. Expression patterns will be confirmed by Northern analysis of wounding time-courses. Detailed sequence analysis will be performed in order to predict the functions of the wound-induced predicted peptides.

F-3 Thesis Outline

The materials and methods are listed (Chapter 2). The construction and differential screening of the FTC-induced systemic poplar leaf SSH library are described (Chapter 3). Detailed sequence analysis of the identified induced clones

was performed to identify the gene products (Chapter 3). Induction locally and systemically was confirmed in poplar leaves after mechanical wounding (Chapter 4). Induction of gene expression following herbivory was compared to mechanical wounding and the chemical elicitors MeJa and SA (Chapter 5). Additionally, unwounded poplar tissues were examined to explore developmental gene expression of the wound-induced genes (Chapter 5). Finally, interesting novel genes with potential defensive functions are discussed (Chapter 6).

CHAPTER 2

MATERIAL AND METHODS

A. MATERIALS AND METHODS

A-1 Overview

The following materials and methods were used to isolate novel poplar genes expressed after FTC herbivory. Systemically FTC-induced poplar leaves were used to create an SSH library. From this library, cDNA clones were PCR amplified and spotted on replica macroarrays for differential screening. Radio-labeled cDNA probes representing total mRNA were used to differentially screen the macro-arrays. The cDNAs showing wound-induction during the macroarray analysis were sequenced. Database homology searches were performed to ascribe potential function to the predicted peptide sequences. Relevant sequences were selected for Northern analysis in wounding time-courses and a tissue-survey to further study gene expression. A diagrammatic overview of all procedures can be seen in Figure 2-1.

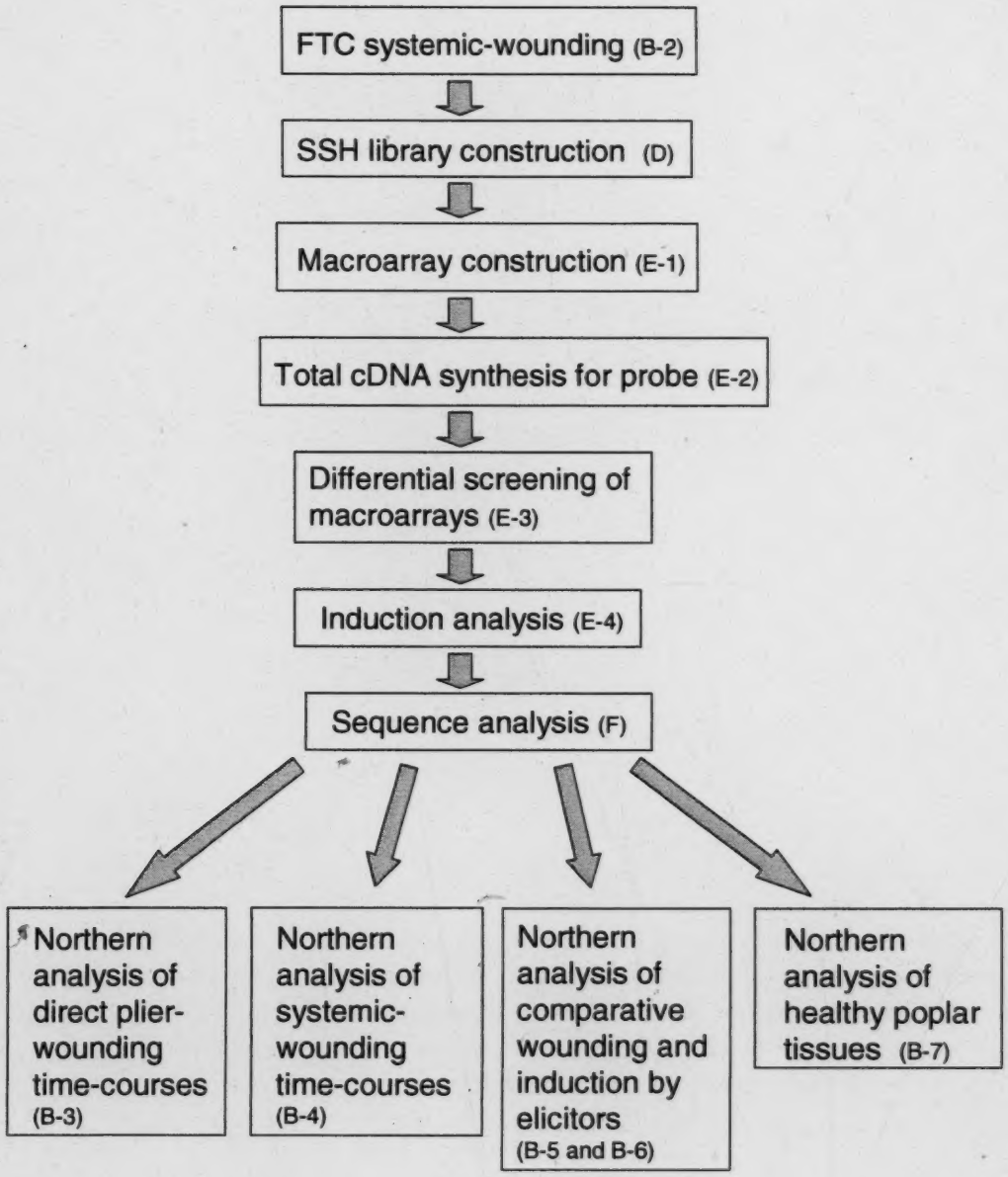
B. PLANT TREATMENTS

B-1 Plant Material

Poplar hybrid H11-11 (*Populus trichocarpa* X *Populus deltoides* [TD]) were obtained from the WSU / UW poplar breeding program. Poplar trees were propagated from hardwood or greenwood cuttings in the University of Alberta Biotron's environmental chambers under 16-h days at 18°C and 75% relative humidity. The soil, lighting, and fertilizer regime were maintained as previously described by Constabel *et al.* (2000). All side shoots were pruned as they developed so that each plant consisted of a single main stem. Plants were approximately 11-weeks-old when used for experiments. The most apical unfolded, but not yet expanded, leaf was

Figure 2-1: Outline of Experiments

An outline of the major experiments presented in this thesis. Letters and numbers in brackets refer to the relevant section of materials and methods.


FTC systemic-wounding (B-2)
SSH library construction (D)
Macroarray construction (E-1)
Total cDNA synthesis for probe (E-2)
Differential screening of macroarrays (E-3)
Induction analysis (E-4)
Sequence analysis (F)
Northern analysis of direct plier-wounding time-courses (B-3)
Northern analysis of systemic-wounding time-courses (B-4)
Northern analysis of comparative wounding and induction by elicitors (B-5 and B-6)
Northern analysis of healthy poplar tissues (B-7)

designated as leaf 1. The other leaves were numbered sequentially down the stem. Leaves below leaf seven were fully expanded.

B-2 Systemic-induction by FTC for Library Construction

Herbivore treatments using FTC were carried out in 75-cm-high insect cages within the environmental chambers. FTC were obtained from Dr. Andrew Keddie or Dr. Jens Roland, University of Alberta. Vigorous 4^{th} and 5^{th} instar FTC were allowed to feed 3X on leaves 6-12 with a 45 min break between feedings. Leaves were harvested 24 h after the commencement of the first feeding. The leaves were grouped in pairs by leaf age starting at leaves 3-4 (leaf pairs: 3-4, 5-6, 7-8, 9-10, 11-12). The midveins were excised with a razor and the tissue sets frozen in liquid nitrogen and stored at -80°C until used. Due to variations in the extent of damage caused by feeding FTC between experiments, samples from different trees were not pooled.

B-3 Mechanical Wound-induction Time Courses

B-3.1 Early Wound-induction (6 h)

For mechanical wounding experiments, all sampled leaves were wounded (leaves 3-11) around their margins with a pair of pliers to simulate herbivory. Wounding was repeated twice at 45 min intervals. Each leaf tissue set consisted of three adjacent leaves starting at leaf 3 (leaf sets: 3-5, 6-8, 9-11). Samples for each time point (0, 1.5, 3, 6 h) were pooled from two trees. The midveins were excised with a razor and the tissue sets frozen in liquid nitrogen and stored at -80°C until used.

B-3.2 Late Wound-induction (48 h)

All sampled leaves were wounded with pliers around their margins as before. Wounding was repeated three times at 1 h intervals. Each leaf tissue set consisted of three adjacent leaves starting at leaf number three, as before. Each time point (0, 6, 12, 18, 24, 36, 48 h) represents samples pooled from two trees. Samples were prepared and stored as before.

B-4 Systemic-induction by Mechanical Damage

Unwounded upper leaves were examined for systemic-induction after mechanical damage as follows. Systemic-induction by mechanical damage was performed by wounding six fully expanded leaves with pliers (9-14) directly below the designated systemic leaves (3-8). Mechanical wounding of leaf margins with pliers was repeated three times with 1 h intervals. Each leaf tissue set consisted of three adjacent leaves starting at leaf number three, as before. Leaves were prepared and stored as before.

B-5 Comparative Wound-induction

In order to compare FTC damage to different types of mechanical damage the following experiment was performed. Herbivore treatments using FTC were carried out as before within cages in the environmental chambers. FTC were restricted to feeding on leaves 9-11 through constant supervision and relocation. As herbivory progressed, the extent of the damage was mimicked on separate trees using a hemostat (surgical pliers) and a hand held hole-punch. Due to variation in FTC

feeding between experiments, each time point was represented by a single tree. Leaves were sampled, prepared and stored as before.

B-6 Induction by Exogenous MeJa and SA Application

The effectiveness of chemical elicitors for wound-induction was tested as follows. MeJa (Bedoukian Research, Danbury, CT) was diluted 1:10 with 95% (v/v) ethanol, and then rediluted 1:500 with: 0.1% (v/v) Triton X-100 in water. Five microliters of 100 mM salicylic acid was diluted 1:20 with water: 0.1% (v/v) Triton X-100 in water. Leaves were sprayed twice to the point of runoff with a 2 h interval. Controls were mock-sprayed with the same solution without MeJa or SA. Leaves were sampled, prepared, and stored as before.

B-7 Poplar Tissue Survey

The leaves of one poplar H11-11 clone were sequentially labeled from the apex. Tissue sets of the leaves and associated petioles were separated and stored for analysis. Midveins of all unfolded leaves were excised with a razor. The apical meristem included the youngest unfolded leaves. The lateral buds were excised from next to the petioles from leaves numbered 6-14. Bark from the stem and main root (main stem below ground) was removed using razor. The large roots were approximately 5 mm in diameter, and the small roots were less than 2 mm and grew at the bottom of the plant. The sucker roots grew near the surface at the outer edge of the pot. Three senescent leaves from the bottom of three different H11-11 clones were

pooled. All tissues were immediately frozen in liquid nitrogen and stored at -80°C until used.

C. RNA PROTOCOLS

C-1 RNA Isolation

RNA was extracted as described in Haruta *et al.* (2001). RNA quality and quantity was confirmed using a spectrophotometer and by ethidium bromide (EtBr) ultraviolet-visualization of electrophoresed samples on a 1% agarose gel. PolyA+ was extracted using a PolyAtract mRNA Isolation Kit (Promega, Madison, WI) without any modifications to the protocols. Quantity and quality of the polyA+ RNA was confirmed using a spectrophotometer.

C-2 Northern Analysis

For Northern blot analysis, seven micrograms of total RNA per lane was separated on a 1% (w/v) agarose-formaldehyde gels in MOPS buffer (pH 7) (0.4 M MOPS [3-(*N*-morpholino)-propanesulfonic acid], 100 mM NaOAc-$3H_2O$, 10mM Na_2EDTA [disodium ethylenediaminetetraacetic acid] and transferred by capillary blotting onto Zeta-Probe membranes (Bio-Rad, Hercules, CA) using standard protocols (Sambrook *et al.*, 1989). The samples were prepared by mixing 6.5 µl of RNA sample with: 2.5 µl of 1X MOPS, 12.5 µl of deionized 50% formamide, 4.5 µl of 2.2 M formaldehyde, and 1 mg/ml EtBr. The samples were heated for 15 min at 65°C and quenched on ice for 5 min. Three microliters of 10X loading buffer added prior to loading. One microgram of *Hind*III digested lambda marker was used as a loading control. RNA quality and quantity was confirmed using a spectrophotometer

and by ethidium bromide (EtBr) ultraviolet-visualization of the electrophoresed samples prior to transfer. The transfer was performed using 10X SSC (sodium chloride/sodium citrate) buffer onto a Zeta-Probe membrane presoaked in 2X SSC. RNA was fixed to the membrane with UV using a GS Gene Linker UV chamber (Bio-Rad). Prehybridization was performed for 2 h at 42°C in 5X sodium chloride/sodium phosphate/EDTA (SSPE), 50% (v/v) formamide, 5X Denhardt's solution, 1% (w/v) SDS, 10% (w/v) dextran sulfate, and 100 mg mL1 denatured salmon sperm DNA. DNA probes were obtained by random priming (T7 Quickprime kit, Pharmacia Biotech, Piscataway, NJ) and hybridization carried out for 16 to 18 h. The membranes were washed twice with 5X SSPE and 1% (w/v) SDS for 15 min at room temperature, twice with 1X SSPE/1% (w/v) SDS at 65°C for 30 min, and once with 0.1X SSPE/1% (w/v) SDS at 65°C for 30 min. Hybridization blots were autoradiographed using a PhosphorImaging system (Molecular Dynamics, Sunnyvale, CA).

C-3 Regression Analysis of the Late Wound Response Time-course

The densitometry values for the selected genes were normalized using actin and calculated using ImageQuant (Molecular Dynamics). A linear regression analysis was performed using Microsoft Excel using the default parameters. The P (probability) value was calculated at the 95 % threshold. Genes showing a continual increase in expression over time will have the strongest correlation while those genes that show an early peak and a following gradual decline will not show correlation of wound-induction over time. This is a limitation of a non-exponential linear analysis.

D. SUPPRESSIVE SUBTRACTIVE HYBRIDIZATION (SSH) cDNA LIBRARY

D-1 SSH-cDNA Construction

The SSH library was made using a Clontech PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA). A brief overview of the SSH technique can be seen in Chapter 1, Figure 1-3. After consulting with the manufacturer, the first strand synthesis was performed using four times the amount of polyA+ RNA recommended in the protocol. No other changes were made to the protocol. The tester (induced sample) was made from FTC systemically-induced leaves 3-4. The driver (control) sample was from unwounded leaves numbered 3-4.

D-2 Cloning SSH-cDNA into pBluescript

Prior to cloning, the amplified subtracted cDNA was visualized under ultraviolet light after staining with EtBr revealing a smear of products ranging in size from 1.5 kb to 200 bp, with the majority of the PCR product between 200-500 bp as expected. Several unsuccessful attempts were made to clone the SSH cDNA products directly into pBluescript. Therefore the cDNA fragments were cloned after separation and extraction from a 1% agarose gel. The gel extracted fragments (Qiaex II gel extraction kit, Qiagen Inc., Mississauga, ON) were cloned into pBluescript-T vector (Marchuk *et al.*, 1991). After blue-white selection, bacterial colonies were screened by PCR for cDNA inserts. Standard T3 and T7 primers corresponding to the region flanking the multiple cloning site of pBluescript SK+ were used to amplify the entire insert from the bacterial colonies. PCR was performed as follows: 95°C 4 min, (95°C

30 sec, 58°C 30 sec, 72°C 1.5 min) 35 cycles (PTC-200 Peltier Thermal Cycler, MJ Research, Waltham, MA) in a 75 µL volume using standard PCR reagents and concentrations (Sambrook *et al.*, 1989). PCR generated bands were separated on 1.2% agarose gels and quantified using AlphaEase software (AlphaEase software v5.1, Alpha Innotech Corporation, San Leandro, CA). The quantified PCR products were used for macroarray construction and as template for sequencing.

E. Differential Screening

E-1 Macroarray Construction

Six replicates of the DNA macro-arrays were constructed by transferring PCR product onto Zeta-probe membranes (Bio-Rad) using a vacuum dot blot apparatus. PCR generated DNA (100 µg) was diluted to a volume of 400 µl with a final concentration of 0.4 M NaOH, 10 mM EDTA. A Zeta-probe membrane (Bio-Rad) and two pieces of Whatman paper were cut to size, pre-wet with dH_20, and mounted in the vacuum blotting apparatus. Under vacuum, the wells were rinsed with dH_20, the DNA samples were loaded, and the wells were rinsed with 500 µl of 0.4 M NaOH, 10 mM EDTA. The membrane was briefly neutralized in 2X SSC, rinsed in dH_20, and the DNA was fixed to the membrane with UV using a GS Gene Linker UV chamber (Bio-Rad).

E-2 Total cDNA Probes for Macroarray Screening

cDNA probes were synthesized from RNA extracted from systemically-induced leaves 3-4 and 5-6 (wounded leaves 9-14). Treatments included FTC-

herbivory, plier-wounding, and unwounded control trees. Total cDNA was made by reverse transcription using a SMART PCR cDNA Synthesis Kit (Clontech) that incorporates adaptors onto both ends of the cDNA. This provides the option of amplifying the cDNA by PCR using the appropriate primers. First-strand cDNA and PCR amplified cDNA were made according to the SMART PCR cDNA Synthesis Kit protocols (Clontech).

E-3 Macroarray Hybridization and Washes

Total cDNA (100-200 μg) was used to make radio-labeled probe. Both first-strand total cDNA and amplified total cDNA were used in separate experiments. Probe was made by random priming (T7 Quickprime kit, Pharmacia Biotech) using twice the recommended [α^{32}P] dCTP (Amersham Pharmacia Biotech, Piscataway, NJ). Pre-hybridization of the macro-arrays was performed in 15 ml buffer containing 5X SSPE (sodium chloride/sodium phosphate/EDTA), 50% (v/v) formamide, 1% (w/v) Denhardt's, 1% (w/v) SDS (sodium dodecyl sulfate, 10% (w/v) dextran sulphate, and 100 ng ml^{-1} denatured salmon sperm DNA, at 42°C for 2 h. Hybridization was carried out in the same buffer, supplemented with freshly denatured probe, at 42°C for 18 h. Membranes were initially washed at room temperature twice for 15 min in 2X SSPE, 0.1% (w/v) SDS. Membranes were then successively washed at 65°C twice for 30 min in 1X SSPE, 0.1% (w/v) SDS, and once for 30 min in 0.1X SSPE, 0.1% (w/v) SDS. Macro-arrays were autoradiographed using a PhosphorImaging system (Molecular Dynamics).

E-4 Calculation of Wound / Control Induction Quotient

In order to calculate wound-induction, autoradiographed macro-arrays were quantified using ImageQuant software (Molecular Dynamics). Four control wells on the array loaded without PCR product were used to calculate the mean background signal. The mean background was then subtracted from the signal intensities measured from each spotted sample. This value was then divided by the signal intensities for each of the house-keeping genes Chlorophyll *ab* binding protein (CAB), rubisco, and malate dehydrogenase to normalize for differences in probe specific activity. These three normalized signals, using CAB, rubisco and malate dehydrogenase, were then averaged to give an average-normalized signal intensity for each spotted cDNA sample on a given blot. The normalized signal for each spotted cDNA from the wounded samples (FTC or plier) was then divided by the corresponding normalized signals from the control leaves to provide a final wound-induction quotient.

E-5 Variation Calculation For wound-induction Replicates

The mean induction, standard deviation, and range were calculated for a randomly selected clone for each of the 17 unique sequences identified. These statistics were calculated using the normalized wound / control induction quotients (see above) with Microsoft Excel.

F. SEQUENCE ANALYSIS

F-1 cDNA Sequencing

DNA for sequencing was purified directly from the PCR reaction (PCR purification kit, Qiagen Inc., Mississauga, ON) or gel purified (Qiaex II gel extraction kit, Qiagen Inc., Mississauga, ON). DNA yields were estimated on EtBr stained electrophoresed gels. Sequencing reactions were performed using either a T3 or T7 primer using the CEQ DTCS sequencing kit (Beckman Coulter, Fullerton, CA) in a thermocycler (96°C 20 sec, 50°C 20 sec, 60°C 4 min) 30 cycles, ethanol precipitated, resuspended in 40 µL formamide and analyzed on a capillary sequencer as per manufacturers instructions (CEQ 2000XL, Beckman Coulter, Fullerton, CA).

F-2 Classification of cDNAs by sequence similarity

CEQ-generated cDNA sequences were manually edited to remove vector and poor quality 3'-sequence and edited to correct ambiguities and miscalled bases. Edited sequences were assembled into contigs using Genetool software (BioTools Inc., Edmonton) using the default settings. The consensus sequences from the contigs were searched against NCBI databases using the TBLASTX program (http://www.ncbi.nlm.nih.gov/BLAST/) in order to determine the predicted coding frame. BCM sequence utilities (http://searchlauncher.bcm.tmc.edu/seq-util/seq-util.html) were used to conceptually translate the nucleotide sequences in six frames. The six translated frames were then used for BLASTP analysis to confirm that only one reading frame encoded a peptide with matches in the database. In addition, BLASTP was used to perform CD (conserved domain) searches. The appropriate

predicted peptide sequences were used as queries with the BLASTP and PSI/PHI-BLAST programs to determine putative identities and functions based on sequence similarity to protein sequences in the NR (non-redundant), PDB (protein database), and CD databases.

Multiple sequence alignments were performed using ClustalW 1.8 (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). BOXSHADE was used to perform identity and similarity shading with the ClustalW 1.8 alignments (BOXSHADE pretty printing- http://www.ch.embnet.org/software/BOX_form.html). Genedoc was used to calculate the percent identity and percent similarity of ClustalW 1.8 aligned sequences (Nicholas, K.B. and Nicholas H.B. Jr. 1997 GeneDoc: Analysis and Visualization of Genetic Variation, http://www.cris.com/~Ketchup/genedoc.shtml).

CHAPTER 3

DIFFERENTIAL SCREENING OF THE SSH LIBRARY AND ANALYSIS OF PREDICTED PROTEINS

A. INTRODUCTION

In order to maximize the chances of discovering novel differentially expressed genes, an SSH library was constructed. This method should decrease the representation of house-keeping genes while simultaneously increasing the representation of differentially expressed genes, thus it increases the chance of discovering a new gene. Herbivory by FTC and mechanical wounding by pliers are both expected to induce wound-responsive genes. To identify genes which might be induced specifically by FTC-herbivory, the SSH library was constructed from RNA extracted from leaves systemically-induced by FTC-herbivory. To minimize the representation of non-defense genes induced for wound repair, systemically-induced leaf tissue was chosen rather than directly damaged leaf tissue. Using known defense genes, TI and PPO, these tissues were shown to be highly induced by herbivory. This chapter describes the results of the macroarray screening process, and analysis of the genes identified. These were characterized and described on the basis of sequence similarity with genes and proteins in the NCBI GenBank databases.

B. RESULTS

B-1 SSH Library

The library contained inserts in the size range of 200-450 bp. Sequencing approximately 226 random cDNA inserts revealed that the library contained some defense genes such as TI. There was also a significant level of redundancy.

B-2 Differential Screening of the SSH Library

Five-hundred and seventy SSH clones were PCR-amplified to provide DNA for the construction of the macro-array. An additional 46 clones of known sequence were included from a parallel H11-11 EST project (Mary Christopher and Lynn Yip, unpublished data). The inclusion of the known 46 clones served the purpose of representing house-keeping control genes and known wound-induced genes (positive controls). Six replica macroarray sets were constructed for differential screening.

For differential screening, the macroarrays were hybridized with labeled cDNA made from the treated tissues: i) FTC-systemically induced leaves, ii) plier systemically-induced leaves and iii) untreated control leaves. The measured signal intensities from the spotted cDNAs after hybridization were adjusted for background signal. To allow comparison between experiments, the different probes were normalized using the house-keeping genes CAB, rubisco, and malate dehydrogenase. A wound / control induction quotient was calculated for each cDNA and treatment. For unknown reasons, a high level of variation in the calculated wound / control induction quotient was seen between replicas. The variation (Table 3-1) made it difficult to determine if there was differential expression between wounding treatments (FTC and plier) and identical inserts were found in each of the fold-induction categories below. For ease of the interpretation of the screen both wounding treatments were used to calculate an average wound-induction.

Of the ~570 SSH clones, a total of 165 were identified as induced 2-fold or more. Thirty cDNAs that showed a 5-fold or greater induction, 44 cDNAs that showed a 3.5- to 5-fold induction, and 91 that showed a 2- to 3.5-fold induction. For

Table 3-1. Calculated Variation for the Macroarray Analyzed Wound-induction

The mean induction, standard deviation, and range were calculated for a randomly selected clone for each of the 17 unique sequences identified. These statistics were calculated using the normalized wound-induction quotients from plier and FTC treatments of leaves 3-4 and 5-6 with Microsoft Excel to demonstrate the wide range of variability in the replicates.

Clone	Putative ID	Mean induction	Standard deviation	Range
F-5	P450	2.8	1.3	3.0
F-8	auxin binging protein	2.0	0.61	1.3
F-13	Pop3	2.0	1.3	2.5
F-35	Cellulose synthase	2.0	1.4	3.4
F-50	60S	2.1	0.70	1.6
F-51	Trypsin inhibitor	5.1	3.3	6.7
F-55	Vegetataive storage protein	2.0	0.58	1.1
F-59	40S	2.1	0.65	1.4
F-63	MADS box	2.2	1.7	3.5
F-64	Apyrase	2.6	1.4	3.3
F-68	Rubisco-small subunit	2.2	0.17	0.40
F-71	Unknown	2.7	0.45	1.0
F-85	Extensin	2.4	1.4	3.3
F-101	Apyrase	3.2	2.1	5.0
F-108	Acid phosphatase	3.1	1.0	2.1
F-211	snRNP	2.2	1.1	2.6
F-229	Lipid transfer protein	2.9	1.0	2.2
F385	Rubisco-small subunit	2.0	1.1	2.4

comparison, TI and PPO showed an average induction for all wounding experiments of 5.1-fold and 1.3-fold respectively. Despite the identification of 165 induced clones, only 17 unique genes were identified revealing the redundant nature of the SSH library.

B-3 Classification of Predicted Peptides by Homology

CEQ-generated cDNA sequences were imported and assembled using Genetool software (BioTools Inc.). Overlapping fragments of genes were joined and redundant sequences were used to create consensus sequences. In some cases, ESTs from a parallel EST project were used to extend or bridge the SSH cDNAs. The consensus cDNA sequences were conceptually translated to predict their reading frames and putative peptides. BLASTP analysis of most of the peptide sequences revealed matches to consensus conserved domains (CDs) in characterized protein families (pfam) as well as matches to proteins with characterized enzymatic activity. Significant matches were obtained using BLASTP for all of the queries except F-55 which were obtained using PSI/PHI-BLAST analysis. PHI-BLAST (Pattern Hit Initiated BLAST) and PSI-BLAST (Position Specific Iterated BLAST) use a pattern or position specific iterative search in which one round of searching is used to build a score model for the next round of searching. This approach is a more sensitive way to identify weak similarities that are often missed by BLASTP. The best matches and scores from the NR database are reported in Table 3-2 and include the genus, accession number, and name of the match. Another method of measuring relatedness is shared amino acid identity (% ID) and amino acid similarity (% SIM) the latter of

Table 3-2: Sequence Analysis of Poplar Genes

Sequence analysis of genes from hybrid poplar H11-11 identified during differential screening of the SSH library, or directly selected from a hybrid poplar EST collections. The clone naming system is explained in text in section B-3. The genes are categorized as to previously identified or known wound-induced poplar genes, genes not previously known to be wound-induced in poplar, or not wound-induced. The last category includes genes analyzed for comparisons, as well as genes identified here which turned out not to be induced by wounding. These genes were identified as to putative biochemical roles based on similarity (e-values) to proteins with demonstrated function or to conserved domains of protein families (pfam). The accession no. and species of the best database match is also given.

	Clone used as probe	Query length (AA)[a]	Best database match: Genus (accession no.) gene[b]	Identity score: Score (e-value)[c]	Conserved Domain: pfam[d]	Identity Score: Score (e-value)[c]
Previously identified wound-induced genes	H1506	316	*Brassica* (AAA32986): endochitinase CH25 precursor	301 (6e-81)	pfam00182: Glyco_hydro_19, chitinase class I pfam00187: Chitin_bind_1, chitin recognition portein	365 (2e-102) 56.8 (1e-09)
	PtdPPO	563	[ef]*Malus* (P43309): polyphenol oxidase	570 (e-161)	pfam00264: tyrosinase, common central domain	55.2 (6e-09)
	F-51	201	[g]*Salix* (AAA68962): Kunitz protease inhibitor	215 (1e-55)	pfam00197: Kunitz_legume, Trypsin and protease inhibitor	125 (1e-30)
	F-55	322	*Populus* (AAK01124): vegetative storage protein	472 (2e-132)	pfam01048: PNP_UDP_1, phosphorylase family	125.0 (3e-04)
	H2339	318	*Arabidopsis* (NP_567699): predicted protein	198 (7e-50)	pfam01048: PNP_UDP_1, phosphorylase family	53.3 (1e-08)

Novel wound-induced genes	F-71	68	*Arabidopsis* (AAL11546): Unknown protein	107 (2e-23)	none	N/A
	F-13	108	*Populus* (T09703): pop3	203 (2e-52)	none	N/A
	F-3.4.11	113	*Populus* (S30515):wound-induced protein	221 (9e-58)	none	N/A
	F-5	224	[c]*Nicotiana* (AAK62346): 5-epi-aristolochene-1,3-dihydroxylase	293 (9e-79)	pfam00067: p450, cytochrome P450	190 (9e-50)
	F-108	261	[c]*Glycine* (T07086): acid phosphatase	306 (1e-82)	pfam03767: acid_phosphat_B, acid phosphatase (class B)	216 (8e-58)
	F-64	92	[c]*Dolichos* (AAD31285): apyrase	111 (2e-24)	pfam01150: GDA1_CD39, nucleoside phosphatase	65.5 (1e-12)
	F-101	159	[b]*Dolichos* (AAF00610): apyrase-2	181 (2e-45)	pfam01150: GDA1_CD39, nucleoside phosphatase	114 (4e-27)

Genes not induced by wounding	H592	111	*Elaeis* (AAK84834): actin-1	234 (9e-62)	pfam00022: actin, actin 1	236 (4e-64)
	H1756	324	*Arabidopsis* (P46488): basic endochitinase	454 (e-127)	pfam00182: Glyco_hydro_19, chitinase class I	187 (5e-49)
	H881	321	*Gossypium* (AAD29050): carbonic anhydrase	423 (9e-118)	pfam00484: Pro_CA, carbonic anhydrase	303 (1e-89)
	H1907	164	*Zea* (AAG34818): glutathione S-transferase	148 (3e-35)	pfam02798: GST_N, N-term domain	65.7 (8e-13)
	F-1.6.7	41	*Gossypium* (T09790): lipid transfer protein	49.7 (6e-06)	pfam00279: plant LTP, lipid transfer protein	42 (7e-06)
	H358	154	*Cucumis* (P46488): malate dehydrogenase	211 (3e-55)	pfam00056: ldh lactate/malate dehydrogenase	100 (5e-23)
	F-2.6.6	88	*Gossypium* (AAL67991): RD22-like: dehydration responsive protein	146 (4e-35)	pfam03181: BURP_domain, unknown function	110 (9e-27)

[a]Conceptual translation of the nucleotide sequence.
[b]Sequence with the highest score according to NCBI BLASTP program.
[c]Identity score according to the NCBI BLASTP program.
[d]Conserved domains with the closest match according to the NCBI BLASTP program.
[e]Sequences with characterized enzymatic function.
[f]Populus PPO has characterized enzymatic function.
[g]Aspen TI has characterized bovine-Trypsin inhibition activity.
[h]Very strong sequence homology found to an apyrase with characterized enzymatic function: Arabidopsis (AAF66599).
[i]Identity score according to the NCBI PSI-BLAST program.

which accounts for conserved changes. Note that these values were often calculated from short fragments of the genes (see column 2 of Table 3-2).

Some of the sequences analyzed from the SSH library were not selected based on the differential screen. Instead, they were selected based on sequence novelty and are denoted differently to distinguish them. Genes identified on the basis of differential screening from the SSH library are denoted by "F-" followed by a number, for example F-108. A number of cDNAs that had been identified by sequencing as part of the library characterization are denoted by an "F-" followed by a glycerol stock identification number such as F-3.4.11. In addition, a detailed analysis of several genes from a parallel EST sequencing project from a separate (wound-induced) library were included in the analysis for comparative purposes and are denoted by an "H" followed immediately by a clone number, for example H523.

B-3.1 Previously Identified Wound-induced Genes in *Populus*

This analysis identified several previously isolated wound-induced genes from poplar and these were included in the protein sequence analysis. These analyses provide a baseline to compare the novel sequences to regarding homology scores (score and e-value) and similarity scores (% ID and % SIM).

H1506: Acidic Endochitinase (*win8*)

H1506 encodes a fragment of the poplar acidic endochitinase *win8* (AAA96702). The endochitinase is considered acidic due to its predicted isoelectric potential. Endochitinases catalyze the hydrolysis of the 1,4-beta-linkages of N-acetyl-D-glucosamine polymers of chitin. The predicted peptide has 41% ID and 53% SIM

to the consensus sequence of chitinase class I pfam00182. The *win8* N-terminus shows 68% ID and 71% SIM to the chitin binding domain consensus sequence pfam00187 (Figure 3-1).

PtdPPO: Polyphenol oxidase:

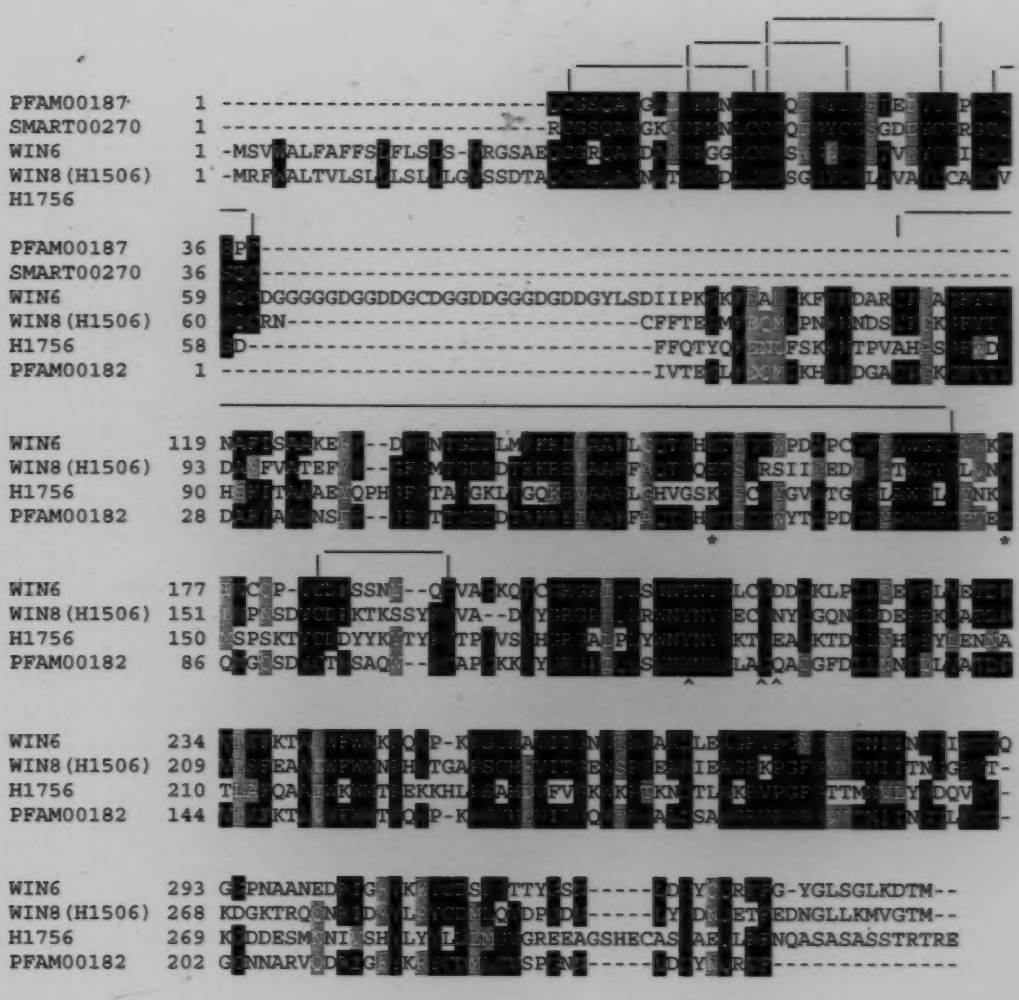
PtdPPO is the full length poplar PPO (AAG21983) that was previously characterized in detail (Constabel *et al.*, 2000). PPO oxidizes *ortho*-hydroxyphenolic compounds to quinones and is known as an antinutritive defense. PPO belongs to pfam00264 (24% ID and 38% SIM) which contains the common central domain of tyrosinase (Table 3-2). In addition to tyrosinases and PPOs, pfam00264 also contains some distantly related hemocyanins. The inclusion of such diverse proteins into the pfam may have weakened the similarity between the pfam concensus and PPO. A concensus containing only PPOs and not other related proteins should be significantly more similar to the poplar PPO than is pfam00264.

F-51: Trypsin inhibitor-*win3.12*

Clone F-51 represents a previously characterized wound-induced TI, TI-*win3.12* (S77703) (Hollick and Gordon, 1993). This clone was one of 14 redundant TI sequences isolated from the SSH library. TI-*win3.12* is related to pfam00197 (Table 3-2) legume Kunitz-type Trypsin inhibitors and shares a relatively low 30% ID and 43% SIM to the consensus sequence. TI-*win3.12* is related to aspen TI2 (55 %ID and 71 %SIM) which has demonstrated inhibition of bovine trypsin (Haruta *et al.*, 2001). TIs are well-established antiherbivore defenses of plants.

Figure 3-1: Protein Sequence Alignment of Poplar Endochitinases

Alignment of deduced amino acid poplar endochitinase sequences of a novel class IB family 19 endochitinase, H1756, and two poplar class IA family 19 endochitinases *win6* (P16579) and (H1506)-*win8* (M25337). Amino acid identity is indicated in black and similarity by gray shading. Disulphide-bonds were identified by similarity to known family 19 endochitinases and are indicated above the sequences, (*) indicates the catalytic glutamates, (^) indicates residues thought to be involved in substrate binding.


PFAM00187
SMART00270
WIN6
WIN8(H1506)
H1756
PFAM00182

F-55 and H2339: Vegetative storage proteins

F-55 represents a full length coding sequence that corresponds to a partial sequence PNI288 (AAK01124) in the NCBI database, a recently identified vegetative storage protein. H2339 corresponds to a previously identified VSP-*win4.5* (S39502). These VSPs belong to a large family of vegetative storage proteins in poplar that contains a bark storage proteins (BSPs), and VSPs such as *win4.5* and PNI288 (Lawrence *et al.*, 2001). F-55 is considered a novel sequence here because F-55 belongs appears to represent the complete coding sequence of a previously published partial clone, PNI288 (Lawrence *et al.*, 2001). An in-depth analysis of both VSP sequences revealed a previously unidentified enzymatic domain. The full length VSP sequences, *win4.5* and F-55, have similarity to phosphorylases of pfam01048 (Table 3-2). F-55 shares 12% ID and 24% SIM with the consensus sequence of pfam01048. The *win4.5* gene is more similar to pfam01048 and shares 17% ID and 27% SIM. In comparison, F-55 and *win4.5* share 41% ID and 61% ID. The sequence similarity of *win4.5* and F-55 to pfam01048 extends the length of the protein (Figure 3-2) with the exception of the N-terminal vacuolar targeting sequence (Coleman and Chen, 1992) which is not included in pfam01048.

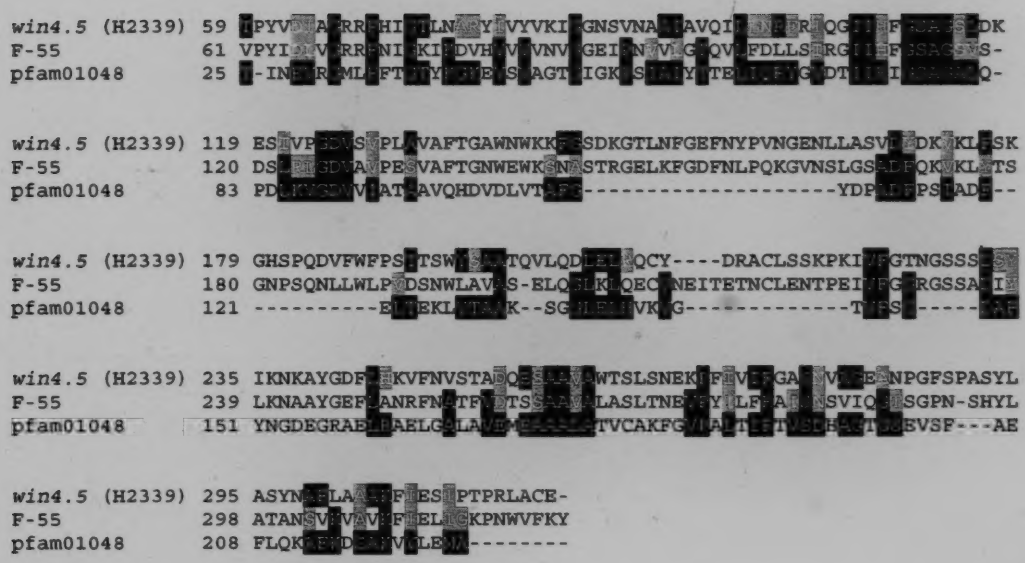
B-3.2 Novel Wound-induced Genes in *Populus*

F-71

Database searches reveal that F-71-like sequences are common and that there are nearly 200 related plant ESTs with homology to F-71. However there is only one predicted protein, in *Arabidopsis* (AAL11546), in the NCBI non-redundant database.

Figure 3-2: Sequence Alignment of Poplar Vegetative Storage Proteins

Multiple sequence alignment of the predicted amino acid sequences of vegetative storage protein H2339, full length = VSP-***win4.5*** (accession no. S39502), and **F-55** (PNI288-like), and the phosphorylase conserved domain **pfam01048**. Residues identical with pfam01048 are shown in black; similar residues are shown in grey. Alignments were performed using ClustalW 1.8 multi-alignment tool (searchlauncher.bcm.tmc.edu) and Boxshade (www.ch.embnet.org/software/BOX_form.html).



It is possible to have 200 ESTs without predicted peptides because the open reading frame for F-71-like sequences are only about 210 nucleotides long encoding a protein less than 70 amino acids in length. In some of the related ESTs, there are alternate open reading frames of similar size, however these other open reading frames are not conserved. Because the F-71 predicted peptide is so small, most translations that are examined manually or by computer programs will overlook it. The strong conservation of the predicted peptide to other F-71-like sequences allowed F-71 to be identified as a putative protein. F-71 is a small conserved predicted protein with related sequences in monocots (*Zea mays*: AI944163), dicots, gymnosperms (*Pinus taeda*: BG039325), and bryophytes (*Marchantia polymorpha*: AU082034) (Figure 3-3). It is without homology to database pfam conserved domains, and therefore the biochemical function of this gene product is not known.

F-13 and F-3.4.11: Pop3 and Pop3-like sequences

F-13 (Pop3) and F-3.4.11 (Pop3-like) are related sequences (Table 3-2) that share 35% ID and 58% SIM. Clone F-13 is nearly identical to a boiling stable protein (BspA, CAC34953) from *Populus tremula* and is identical with a protein named Pop3 (T09703) from *Populus trichocarpa* X *P. deltoides*. F-3.4.11 and a wound-induced protein (S30515) in *Populus trichocarpa* X *P. deltoides* are also identical. There are two closely related Pop3sequences in *Arabidopsis* (AAK43839 and AAK91994) and there are two more distantly related sequences (NP_180725 and NP_175547) that have a C-terminal extension of about 110 amino acids (data not shown). There are Pop3-related sequences in dicots, monocots (*Oryza sativa*: AU030791), gymnosperms (*Pinus taeda*: A1725022), bryophytes (*Marchantia polymorpha*:

Figure 3-3: Sequence Alignments of F-71 Predicted Protein Sequences

Identical amino acids are shown in black (80%); similar residues are shown in grey. Comparisons of sequences from the dicots hybrid poplar (*Populus trichocarpa* X *P. deltoides*), POPLAR (F-71: EST accession no. AI166837) and *Arabidopsis thaliana*, ARABIDOPSIS (accession no. AAL11546), the monocot *Zea mays*, MAIZE (EST accession no. AI944163), the gymnosperm *Pinus taeda*, PINE (EST accession no. BG039325), and liverwort *Marchantia polymorpha*, LIVERWORT (EST accession no. AU082034). The liverwort EST contains unidentified nucleotides which may account for the differences in the N- and C-termini of that predicted protein.

POPLAR (F71) 1 MT 68
ARABIDOPSIS 1 ML 68
PINE 1 -MT 67
MAIZE 1 -M 67
LIVERWORT 1 ------MASDQA 64

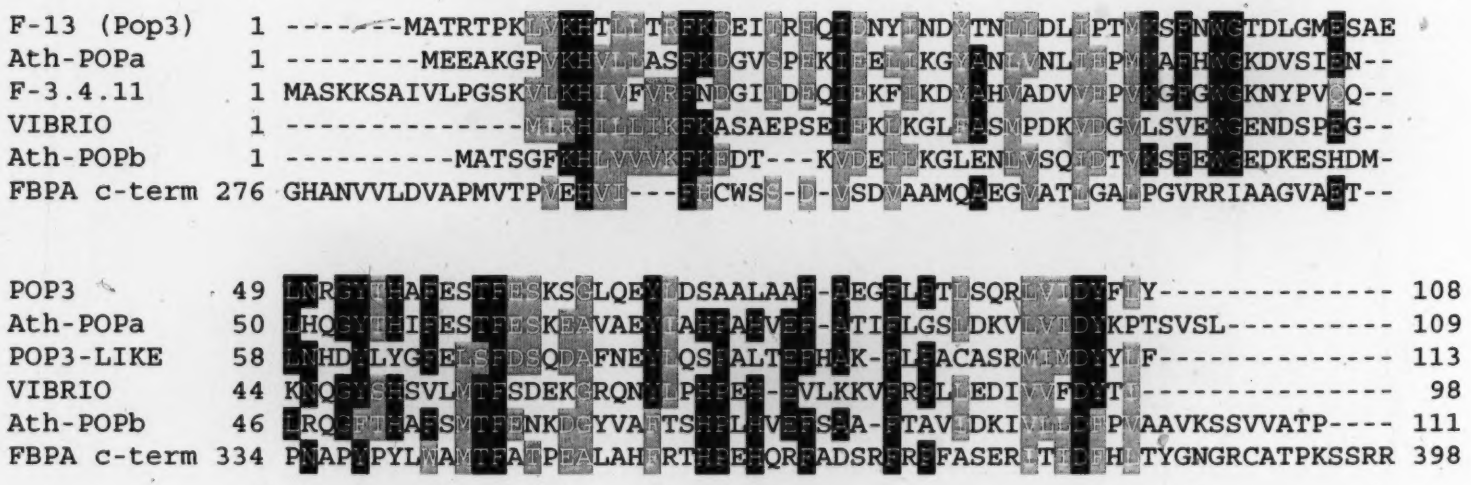
AU082013) (Figure 3-4). BLAST-P analysis revealed that *Vibrio cholerae* has the most closely related non-plant sequence. The *Vibrio* sequence and F-13 share 24% ID and 42% SIM. Since it was unlikely that *Vibrio* would be the only bacteria with a Pop3 related sequence further analysis was done using PSI/PHI-BLAST. Several other bacteria also have Pop3 related sequences, including those from the genus *Agrobacterium*, *Arthrobacterium*, *Chlostridium*, and *Mesorhizobium*. PSI/PHI-BLAST analysis revealed a Pop3 related sequence fused in frame to the C-terminal portion of a fructose 1,6-bisphosphate aldolase (BAA75221) in *Hydrogenophilus thermoluteus*. The Pop3-like C-terminal portion from the fructose 1,6-bisphosphate aldolase shares 14% ID and 32% SIM with F-3.4.11. The Pop3 related sequences do not have conserved cysteines but do share conserved hydrophobic residues (Figure 3-4). None of the Pop3 related sequences have homology to CDs or any proteins domain of known enzymatic function.

F-5: P450

F-5 has homology to cytochrome P450s of pfam00067 (Table 3-2). Hydroxylase, monooxygenase, and reductase activities are among the catalytic activities that P450s perform. The predicted protein fragment has the conserved PERF amino acid motif (data not shown) that is common to P450s (PERF or PDRF). F-5 has very strong homology to an elicitor induced P450 (AAK62346) (Table 3-2) in *Nicotiana* and shares 57% ID and 77% SIM with this sequence. The *Nicotiana* P450 has been cloned and demonstrated to have 5-epi-aristolochene-1,3-dihydroxylase activity (Ralston *et al.*, 2001). F-5 also has strong homology to ferulate 5'

Figure 3-4: Sequence Alignment of Pop3 Related Sequences

Identical amino acids are shown in black (70%); similar residues are shown in grey. Comparisons of sequences from *Populus trichocarpa* X *P. deltoides*, F-13 (Pop3: accession no. T09703) and F-3.4.11 (Pop3-like: S30515), *Arabidopsis thaliana*, Ath-POPa (AAK43839) and Ath-POPb (AAK91494), *Vibrio cholerae*, VIBRIO (AAB81983), and the C-terminal extension of fructose 1,6 bisphosphate aldolase from *Hydogenophilus thermoluteous*, FBPA C-term (B1071535). Alignments were performed using ClustalW 1.8 multi-alignment tool (searchlauncher.bcm.tmc.edu) and Boxshade (www.ch.embnet.org/software/BOX_form.html).


F-13 (Pop3) 1
Ath-POPa 1
F-3.4.11 1
VIBRIO 1
Ath-POPb 1
FBPA c-term 276
POP3 49 108
Ath-POPa 50 109
POP3-LIKE 58 113
VIBRIO 44 98
Ath-POPb 46 111
FBPA c-term 334 398

hydroxylases and flavanoid 3' and 5' hydroxylases involved in phenylpropanoid synthesis (data not shown).

F-108: Acid phosphatase- Vegetative Storage Protein

F-108 has similarity to soybean acid phosphatase vegetative storage proteins VSPα (P15490) (Table 3-2) and VSPβ (P10743) (data not shown), both with glucose 6-phosphatase domains and have demonstrated activity on polyphosphates (DeWald *et al.*, 1992). The soybean VSPs respond to nitrogen status and are regulated by methyl jasmonate, wounding, sugars, light and phosphate (Berger *et al.,* 1995). The relationship of acid phosphatase activity in defense or to a function as a VSP is unknown (see Chapter 6). F-108 shares 60% ID and 76% SIM to VSPα and has 36% ID and 50% SIM to the consensus sequence pfam03767 of class B acid phosphatases.

F-64 and F-101: Apyrase

F-64 and F-101 have sequence similarity to nucleoside phosphatases (apyrases) of pfam01150 (Table 3-2). F-64 shares 24% ID and 46% SIM to a lectin-binding phosphohydrolase (LNP, AAD31286) in *Dolichos biflorus*. The *Dolichos* LNP has demonstrated phosphohydrolase activity and catalyzes the hydrolysis of phosphoanhydride bonds of nucleoside di- and triphosphates (Etzler *et al.*, 1999). F-101 shares 28% ID and 42% SIM to the same LNP homolog (AAF00610) in *Dolichos*. F-64 and F-101 show homology to the pfam01150 family of nucleoside phosphatases. A protein sequence alignment to the pfam01150 consensus sequence revealed that F-64 and F-101 represent different non-overlapping fragments of one or possibly two apyrases (data not shown). There is insufficient sequence data to distinguish between these two possibilities.

B-3.3 Novel *Populus* Genes Not Induced by Wounding

H1756: Basic endochitinase

H1756 is related to chitinase class I pfam00182 (Table 3-2) and shares 36% ID and 54% SIM. H1756 does not have a chitin binding domain at the predicted protein N-terminus typically found in class I chitinases. On the basis of sequence similarity, H1756 is significantly different from the two previous identified chitinases from poplar *win6* (P16579) and H1506-*win8* (M25337) and is missing a proposed catalytic glutamate and has a proline substitution in the substrate binding site (Figure 3-1). It is thus unlikely that the protein has chitinase activity.

H1907: Glutathione S-transferase

Some plant glutathione-S-transferases (GSTs) induced by stress are related to HSP26 which belongs to pfam02798. H1907 is also related to pfam02798 (Table 3-2) and shares 38% ID and 48% SIM to the pfam02798 consensus sequence. GSTs function to conjugate reduced glutathione to a variety of targets that include exogenous and endogenous hydrophobic electrophiles: RX + GLUTATHIONE = HX + R-S-GLUTATHIONE (NCBI: domain search = pfam02798). The glutathione molecule binds in a cleft between N and C-terminal domains; the catalytically important residues are proposed to reside in the N-terminal domain. Insufficient sequence data is available to confirm if the catalytic residues are present.

H881: Carbonic anhydrase

H881 was selected as a possible house-keeping gene. H881 is related to the carbonic anhydrase pfam00484 (Table 3-2) and shares 53% ID and 66% SIM. Carbonic anhydrase is also known as a carbonate dehydratase because is catalyzes the

reversible hydration of carbon dioxide: $H_2CO_3 = CO_2 + H_2O$ (NCBI: domain search = pfam00484).

F-1.6.7: Lipid transfer protein

F-1.6.7 has 51% ID and 63% SIM to the plant LTP consensus sequence pfam00234. Many LTPs in pfam00279 are seed storage proteins that sometimes have trypsin-alpha amylase inhibitor activity (NCBI: domain search = pfam00234) while others are non-specific phospholipids transfer proteins involved in phospholipid transport.

H358: Malate dehydrogenase

H358 was chosen as a house-keeping gene. This clone has 42% ID and 53% SIM to consensus sequence of pfam00056 that includes malate and lactate dehydrogenases.

F-2.6.6: RD22-like

F-2.6.6 was selected for further analysis based on sequence novelty and possible stress responsiveness. F-2.6.6 has 46% ID and 59% SIM to pfam03181 BURP domain. The BURP domain is found at the C-terminus of several different plant proteins. It was named after the proteins in which it was first identified; the BNM2 clone-derived protein from *Brassica napus*; USPs and USP-like proteins; RD22 from *Arabidopsis thaliana*; and PG1beta from *Lycopersicon esculentum* (NCBI: domain search = pfam03181). This domain is around 230 amino acid residues long. It possesses the following conserved features: two phenylalanine residues at its N-terminus; two cysteine residues; and four repeated cysteine-histidine motifs, arranged as: CH-X(10)-CH-X(25-27)-CH-X(25-26)-CH, where X can be any amino

acid. The function of this domain is unknown. RD22 in *Arabidopsis* is responsive to drought (Yamaguchi-Shinozaki and Shinozaki, 1993) but it has no known biochemical function.

C. CONCLUSIONS AND DISCUSSION

From the initial ~570 SSH screened clones, 165 clones were identified as wound-induced by FTC herbivory or pliers. There were 30 cDNAs that showed a 5-fold or greater induction, 44 cDNAs that showed a 3.5- to 5-fold induction, and 91 that showed a 2- to 3.5-fold induction. According to these results nearly 29% of the clones are induced by at least two-fold. Although two-fold may not appear to be a strong induction, it is noted that the wound-induced "marker genes" TI and PPO only demonstrated a 5.1-fold and 1.3-fold induction respectively. Therefore by comparison, an induction of two-fold is a significant induction. It is apparent that the induction-quotients calculated from the macroarray data is compressed compared to the induction measured by Northern analysis, since TI and PPO were induced 30 and 100 fold respectively when measured by Northern blot. This is consistent with previous reports (Schena *et al.*, 1996).

Considering the available data, the cut-off of two-fold induction used to select cDNAs for sequencing will select for strongly induced genes. Genes showing moderate or weak induction will be missed by this method of screening. To compensate for this, some randomly sequenced cDNAs from the same SSH library were also selected for further analysis based on sequence similarity to known wound-induced genes or sequence novelty.

The SSH library construction and differential screening were generally successful. However, the high level of redundancy in the library meant that only 17 unique sequences were identified. Sequence analysis revealed that I had isolated fragments of 11 sequences not previously cloned from poplar, as well as previously sequenced wound-induced genes such as TI-*win3.12* (F-51) and acidic endochitinase-*win8* (H1506). The novel sequences fall into four categories. The first are those that are of unknown function and have no similarity to any known proteins or have similarity to predicted proteins of unknown functions: F-71, F-13, and F-3.4.11. The second category includes genes that appear to be involved in phosphate metabolism and some of which were not known to be wound-induced or defense-related. These include novel genes such as acid phosphatase (F-108), apyrase (F-64 and F-101) and two known VSP genes in poplar (VSP-*win4.5* and F-55). Both VSPs have phosphorylase domains which were not previously identified. The third category of novel sequences has similarity to known proteins in other plants and include a P450 (F-5), a BURP domain containing sequence (F-2.6.6), a glutathione S-transferase (H1907), an endochitinase-like (H1756), and a lipid transfer protein (F-1.6.7). However, Northern analysis later indicated that these were not necessarily wound-inducible in poplar. The final category includes novel sequences that were potential housekeeping genes: carbonic anhydrase (H881) and malate dehydrogenase (H358). In summary, I cloned and sequenced 11 new partial gene sequences from poplar, with potential interaction in poplar defense. This was tested by Northern analysis to confirm induction by real and simulated herbivory (see Chapters 4 and 5).

CHAPTER 4

MECHANICAL- AND SYSTEMIC-INDUCTION OF GENES

A. INTRODUCTION

To confirm the macroarray results and wound-induction after leaf damage, Northern analysis was performed. Using probes corresponding to the genes identified (Table 3-2), poplar leaves were subjected to wounding by pliers to mimic insect damage to examine the spacial and temporal gene expression in wounded leaves. Mechanical-wounding experiments are more consistent than systemic-induction because the area of leaf directly wounded can be controlled whereas the nature of the systemic signal is, at this point, unknown. For this reason mechanical-wounding was used to examine early induction up to 6 h after wounding and late induction up to 48 h after wounding. In addition, to test the possibility that some genes might be induced systemically after damage but not by direct damage, a systemic wounding time-course experiment was performed, where pliers were used to damage lower leaves, but the upper, unwounded leaves were analyzed. These experiments were carried out with as many time points as possible to distinguish possible differences of induction kinetics. In this chapter, the wound-induced expression of the genes identified and described in the previous chapter is presented. The genes are first discussed as categorized on Table 3-2. General trends and highlights are described in the last half of the chapter.

B. RESULTS OF NORTHERN ANALYSIS

B-1 Previously Identified Wound-induced Genes in *Populus*

Because of the importance of developmental leaf age on defense responses (Constabel *et al*,. 2000), leaves of several classes were harvested and analyzed

separately (Figure 4-1). Previously identified wound-induced genes were included in the Northern analysis so that a comparison to the novel genes could be made to determine if their expression profiles were consistent with a defense function. A regression analysis of the induction profiles for the late time course (Figure 4-2) reveals that nearly all of the induction trends are convincing. The summary of the regression analysis is presented in Table 4-1. The induction trends for the systemic time-course are expressed in Table 4-2.

Acidic endochitinase: H1506-win8

H1506 appears to be induced by 3 h at leaf positions 3-5 (Figure 4-1). H1506 showed wound-induction that was consistent regardless of leaf age. Mechanical wound-induction was observable by 18 h (Figure 4-2) and systemic-induction was observable by 12 h (Figure 4-3). The difference in timing is likely due to experimental variation.

Polyphenol oxidase: PtdPPO

PtdPPO appears to be induced in leaves 3-5 by 6 h and perhaps as early as 3 h (Figure 4-1). PtdPPO showed systemic induction that peaked at 24 h (Figure 4-3).

Trypsin inhibitor-*win3.12*: F-51

Trypsin inhibitor is a classic defense gene in many plant model systems. In poplar, F-51 showed very rapid induction, as early as 1.5 h in the young leaves 3-5 (Figure 4-1).

Vegetative storage proteins: H2339 and F-55

H2339 and F-55 appear to be induced fairly late after wounding. H2339 appears to be induced 18-24 h and then abruptly induced again by 36-48 h

Figure 4-1: Early Wound-response Time Course

Early wound-response time course, performed by direct plier wounding to all sampled leaves. Leaf margins were wounded with a pair of pliers twice with a 45 min interval. Each leaf tissue set consisted of three adjacent leaves from positions 3-5, 6-9, 10-12 pooled from two trees at each of the time points: 0 h (unwounded control), 1.5 h, 3 h and 6 h.

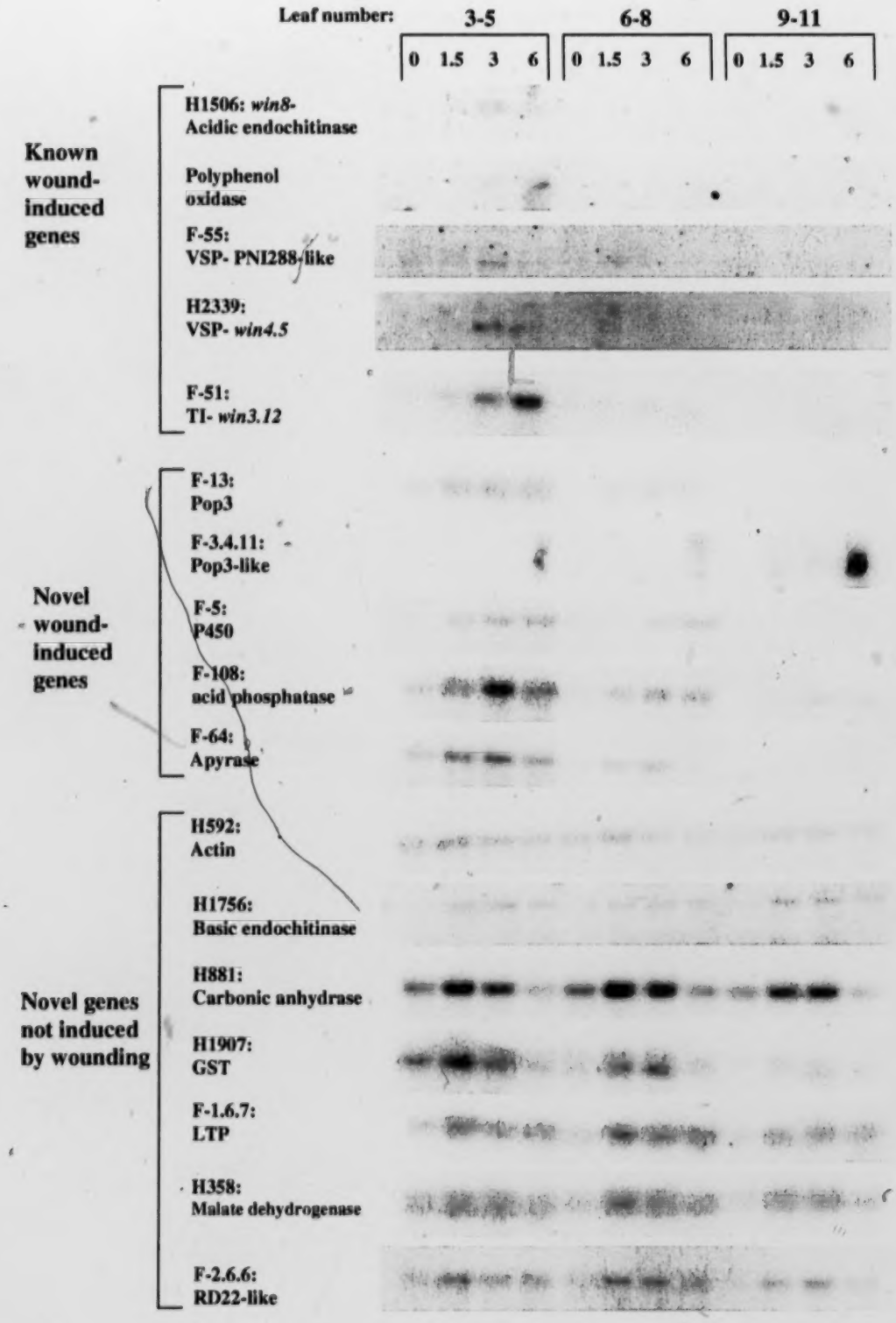

Leaf number:
3-5
6-8
9-11
0 1.5 3 6
0 1.5 3 6
0 1.5 3 6
Known wound-induced genes
H1506: win8- Acidic endochitinase
Polyphenol oxidase
F-55: VSP- PNI288-like
H2339: VSP- win4.5
F-51: TI- win3.12
Novel wound-induced genes
F-13: Pop3
F-3.4.11: Pop3-like
F-5: P450
F-108: acid phosphatase
F-64: Apyrase
Novel genes not induced by wounding
H592: Actin
H1756: Basic endochitinase
H881: Carbonic anhydrase
H1907: GST
F-1.6.7: LTP
H358: Malate dehydrogenase
F-2.6.6: RD22-like

Figure 4-2: Late Wound Response

The 48 h long wound-response time course was performed by direct plier wounding to all sampled leaves. Leaf margins were wounded with a pair of pliers three times with 1 h intervals. Each leaf tissue set consisted of three adjacent leaves from positions 3-5, 6-9, 10-12 pooled from two trees at each of the time points indicated. Hybridization data for leaf positions 3-4 and 5-6 for F-55 were not obtained; only the unobscured samples for leaves 7-9 are included. Similar time courses have been performed two times using different time points and leaf sampling patterns which yielded similar results.

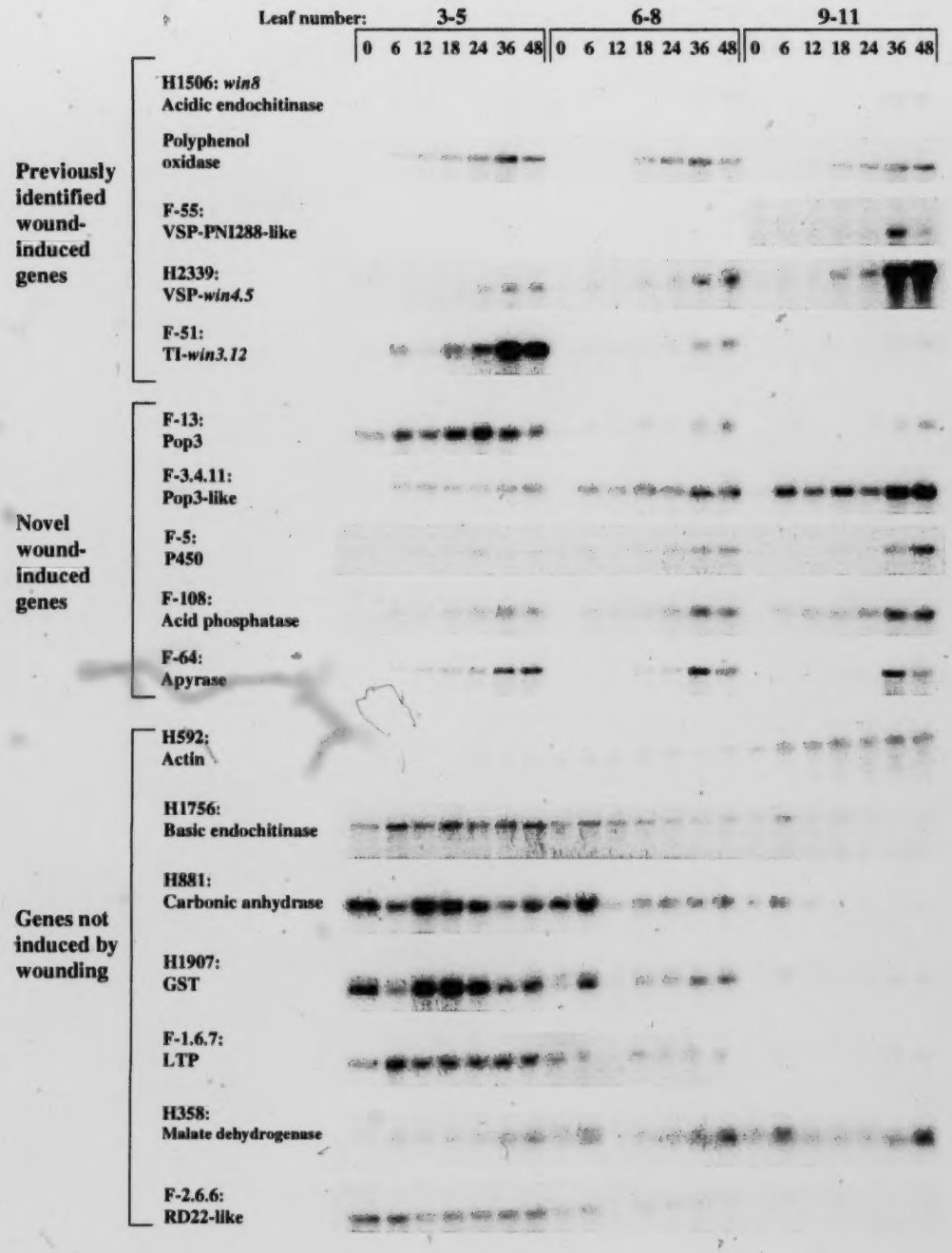

Leaf number:
3-5
6-8
9-11
0 6 12 18 24 36 48 0 6 12 18 24 36 48 0 6 12 18 24 36 48
Previously identified wound-induced genes
H1506: win8
Acidic endochitinase
Polyphenol oxidase
F-55:
VSP-PNI288-like
H2339:
VSP-win4.5
F-51:
TI-win3.12
Novel wound-induced genes
F-13:
Pop3
F-3.4.11:
Pop3-like
F-5:
P450
F-108:
Acid phosphatase
F-64:
Apyrase
Genes not induced by wounding
H592:
Actin
H1756:
Basic endochitinase
H881:
Carbonic anhydrase
H1907:
GST
F-1.6.7:
LTP
H358:
Malate dehydrogenase
F-2.6.6:
RD22-like

Table 4-1. Regression Analysis of the Late Wound Response Time-course

The densitometry values for the wound-induced genes were normalized using actin. The zero time-point was set to a value of one and all other time-points were compared to it. A linear regression analysis was performed using Microsoft Excel. Genes showing a continual increase in expression over time will have the strongest correlation while those genes that show a peak and a decline will show a weak or no correlation of wound-induction over time. The P (probability) value was calculated at the 95 % threshold. P-values below 0.05 and 0.01 are denoted with a single and double asterix respectively.

Clone	Gene expression normalized with actin 0 h	6 h	12 h	18 h	24 h	36 h	48 h	slope	P-value	R square
F-55										
leaves 3-5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
leaves 6-8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
leaves 9-11	1.00	0.66	0.61	0.52	0.56	0.92	0.68	-0.001	NS	0.012
H2339										
leaves 3-5	1.00	1.16	1.12	1.30	1.16	1.74	1.48	0.012	*	0.668
leaves 6-8	1.00	1.02	0.84	1.20	1.24	2.16	2.03	0.028	**	0.801
leaves 9-11	1.00	0.66	0.60	0.72	0.93	1.67	1.73	0.023	*	0.675
F-51										
leaves 3-5	1.00	3.23	2.03	4.12	3.81	6.56	5.25	0.095	*	0.731
leaves 6-8	1.00	0.73	0.73	0.88	1.04	2.51	2.13	0.035	*	0.690
leaves 9-11	1.00	0.56	0.54	0.51	0.57	0.80	0.91	0.003	NS	0.050
F-13										
leaves 3-5	1.00	1.37	1.17	1.50	1.20	1.16	0.75	-0.007	NS	0.227
leaves 6-8	1.00	0.99	0.71	1.35	0.98	2.15	2.01	0.027	*	0.690
leaves 9-11	1.00	1.17	1.21	1.06	1.49	2.28	3.12	0.044	**	0.871
F-3.4.11										
leaves 3-5	1.00	2.40	2.68	2.64	1.72	2.49	2.48	0.016	NS	0.185
leaves 6-8	1.00	2.08	1.84	2.58	2.43	4.11	3.29	0.052	*	0.754
leaves 9-11	1.00	1.67	1.39	1.25	1.17	1.64	1.75	0.010	NS	0.358
F-5										
leaves 3-5	1.00	1.20	1.13	1.22	0.99	1.01	0.84	-0.005	NS	0.417
leaves 6-8	1.00	0.93	0.70	0.91	1.06	1.63	1.39	0.014	*	0.578
leaves 9-11	1.00	0.63	0.62	0.54	0.53	0.84	1.10	0.005	NS	0.130
F-108										
leaves 3-5	1.00	1.83	1.59	1.84	1.52	2.27	1.71	0.013	NS	0.302
leaves 6-8	1.00	1.10	1.01	1.27	1.61	2.96	2.24	0.037	*	0.719
leaves 9-11	1.00	0.97	1.09	0.91	1.16	1.43	1.46	0.011	**	0.771
F-64										
leaves 3-5	1.00	2.74	3.58	4.12	3.25	5.82	5.40	0.011	**	0.771
leaves 6-8	1.00	1.30	0.68	2.48	2.97	7.30	4.66	0.116	*	0.683
leaves 9-11	1.00	0.50	0.55	0.79	0.83	2.88	2.51	0.047	*	0.675

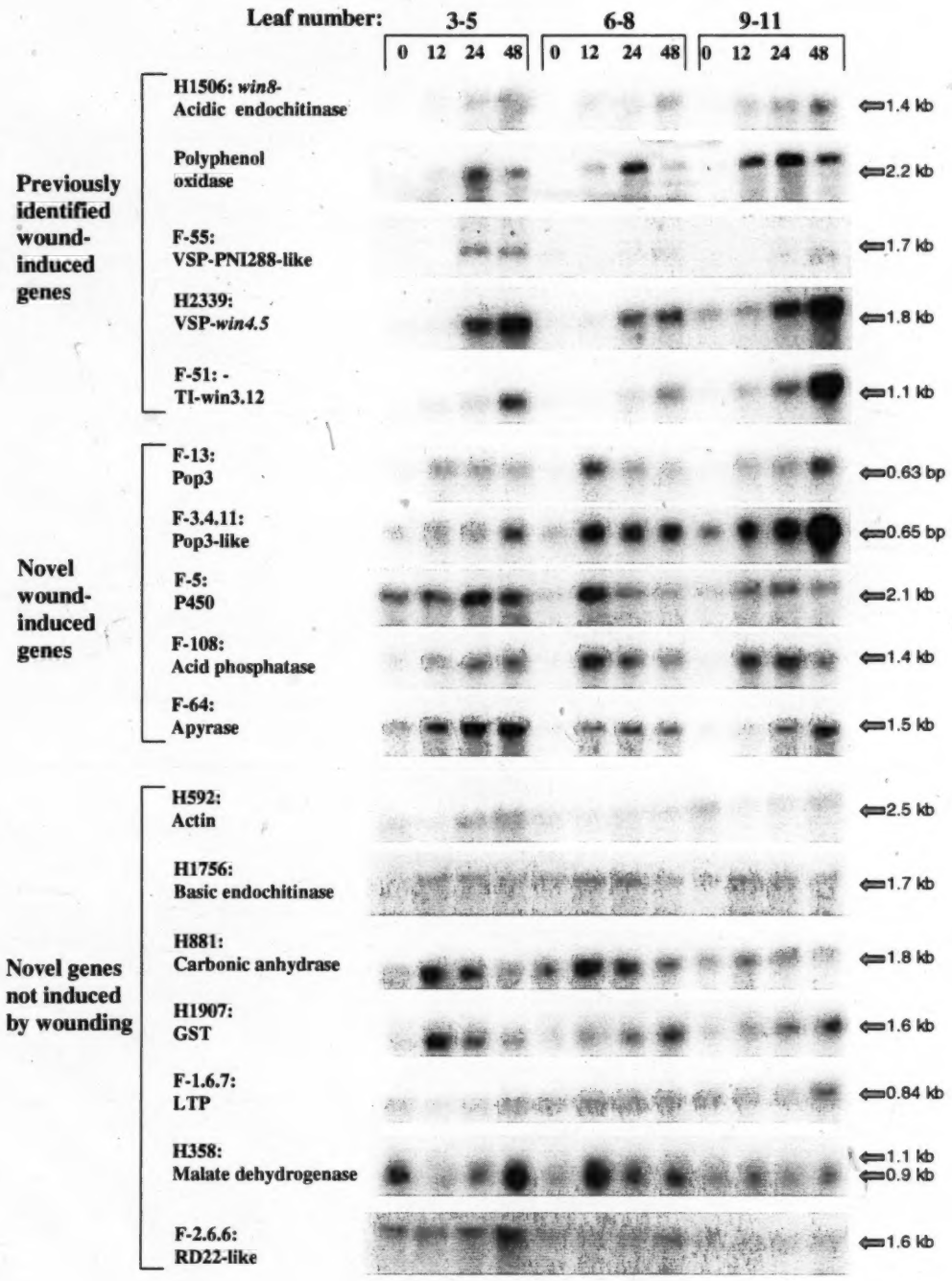
Table 4-2. Trends of Wound-induction After Systemic Induction

The densitometry values for the wound-induced genes in the systemic-induction time-course were normalized using actin. The zero time-point was set to a value of one and all other time-points were compared to it

Clone	0 h	12 h	24 h	48 h
F-55				
leaves 3-5	1.00	1.08	1.51	1.32
leaves 6-8	1.00	1.04	1.00	1.22
leaves 9-11	1.00	1.33	1.40	1.38
H2339				
leaves 3-5	1.00	1.56	2.23	2.33
leaves 6-8	1.00	1.10	1.70	1.82
leaves 9-11	1.00	1.48	1.84	1.82
F-51				
leaves 3-5	1.00	1.94	1.67	2.45
leaves 6-8	1.00	1.15	1.57	2.38
leaves 9-11	1.00	2.19	2.76	3.33
F-13				
leaves 3-5	1.00	2.42	1.60	1.35
leaves 6-8	1.00	2.56	1.71	1.49
leaves 9-11	1.00	2.54	2.54	2.38
F-3.4.11				
leaves 3-5	1.00	1.64	1.06	1.21
leaves 6-8	1.00	1.93	1.55	1.56
leaves 9-11	1.00	1.76	1.83	1.79
F-5				
leaves 3-5	1.00	1.24	0.86	0.75
leaves 6-8	1.00	2.06	1.41	1.16
leaves 9-11	1.00	2.04	1.81	1.22
F-108				
leaves 3-5	1.00	1.66	1.34	1.24
leaves 6-8	1.00	3.04	2.22	1.94
leaves 9-11	1.00	3.10	2.87	1.69
F-64				
leaves 3-5	1.00	1.83	1.44	1.31
leaves 6-8	1.00	2.14	1.92	1.76
leaves 9-11	1.00	2.09	2.87	2.70

Figure 4-3: Systemic Wound-response Time Course

Systemic wound-response time course performed by direct plier wounding six leaves at positions 13-18. Leaf margins were wounded with a pair of pliers three times with 1 h intervals. Each leaf tissue set consisted of three adjacent leaves from positions 3-5, 6-9, 10-12 pooled from two trees at each of the time points indicated. The approximate transcript sizes are indicated by arrows and are calculated from these blots. Similar time courses have been performed two times using different time points and leaf sampling patterns which yielded similar results.


Leaf number:
3-5
6-8
9-11
0 12 24 48 0 12 24 48 0 12 24 48
Previously identified wound-induced genes
H1506: win8- Acidic endochitinase
Polyphenol oxidase
F-55: VSP-PNI288-like
H2339: VSP-win4.5
F-51: - TI-win3.12
Novel wound-induced genes
F-13: Pop3
F-3.4.11: Pop3-like
F-5: P450
F-108: Acid phosphatase
F-64: Apyrase
Novel genes not induced by wounding
H592: Actin
H1756: Basic endochitinase
H881: Carbonic anhydrase
H1907: GST
F-1.6.7: LTP
H358: Malate dehydrogenase
F-2.6.6: RD22-like
⇐1.4 kb
⇐2.2 kb
⇐1.7 kb
⇐1.8 kb
⇐1.1 kb
⇐0.63 bp
⇐0.65 bp
⇐2.1 kb
⇐1.4 kb
⇐1.5 kb
⇐2.5 kb
⇐1.7 kb
⇐1.8 kb
⇐1.6 kb
⇐0.84 kb
⇐1.1 kb
⇐0.9 kb
⇐1.6 kb

(Figure 4-2). The apparent developmental expression in older leaves seen in Figure 4-2 is inconsistent with other experiments and is likely an artifact. Systemically, H2339 appears to be induced by 12 h and abruptly induced at 24 h (Figure 4-3). Similar trends were seen in similar time-courses. F-55 also appears to be induced late. F-55 appears to be induced by 18 h and then appears to be induced abruptly by 36 h in leaves 9-11. Similar trends were seen in replicate blots. Radiolabeled-background obscured the remainder of the blot which is not included (Figure 4-2). Systemically, F-55 appears to be abruptly induced at 24 h with a leaf-age expression bias for younger leaves (Figure 4-3).

B-2 Novel Mechanically- and Systemically-induced Genes in *Populus*

Pop3-like sequences: F-13 and F-3.4.11

F-13 appears to be induced early in leaves 3-5 by 1.5 h (Figure 4-1) although there is background gene expression at 0 h. F-13 showed greater systemic and mechanical-induction in younger leaves than it did in older leaves in some experiments.

F-3.4.11 appears to be induced by 1.5 h in leaves 9-11 (Figure 4-1) and appears to be induced in all the examined leaves by 6 h. F-3.4.11 was systemically-induced in all leaves examined but showed the strongest induction in leaves 9-11 (Figure 4-3). Interestingly, F-13 showed greatest wound-induction in developing leaves while F-3.4.11 was consistently more strongly induced in older leaves.

P450: F-5

F-5 appears to be induced by 1.5 h in the young leaves 3-5 (Figure 4-1). As the leaf age increased the induction was delayed. In leaves 5-6 induction occurred by 3 h (Figure 4-1) and in leaves 9-11 induction occurred by 18 h (Figure 4-2). Although F-5 showed the strongest expression in older leaves in Figure 4-2, similar blots with different leaf sampling patterns revealed that F-5 is usually expressed more strongly in the developing leaves.

Acid phosphatase (VSPα-like): F-108

F-108 appears to be induced in all leaf positions examined by 1.5 h (Figure 4-1), and was among the most rapidly induced genes. F-108 was induced in all the leaf ages examined by both mechanical- and systemic wounding.

Apyrase: F-64 and F-101

Northern analysis revealed that F-64 and F-101 hybridized to transcripts of the same size: 1.5 kb. Northern analysis using both of these cDNA as probes revealed that the bands were super-imposable in both size and intensity. It was assumed that F-64 and F-101 hybridized to the same transcript. The following results present only the results using F-64. F-64 appears to be induced in positions 3-5 and 6-8 by 1.5 h, which like F-108 was very rapid (Figure 4-1). F-64 was strongly induced in all leaves but appears to be induced more strongly in younger leaves than in older leaves.

B-3 Novel *Populus* Genes Not Induced by Wounding

Although H1756 appears to code for an endochitinase it was not systemically- or mechanically- induced. Carbonic anhydrase (H881), LTP (F-1.6.7), Malate

dehydrogenase (H358), and RD22-like (F-2.6.6) were not systemically- or mechanically- induced. However, several genes of this group showed greater expression in the younger leaves.

C. SUMMARY AND DISCUSSION

Of the original 17 unique sequences identified as wound-induced by the macroarray screen, eight appear to be induced by mechanical wounding on Northern blots (this includes F-55 and both apyrases F-64, F-101). All also appears to be induced systemically, and all except F-3.4.11 tended to be induced the greatest in younger leaves. Despite some plant-to-plant variation in kinetics, there were some general trends and differences in different genes in the inducible pattern of expression. Several genes are distinctly early, in particular, F-108, F-64, and to some extent F-51 which may suggest a role in defense signaling. Other genes appear to be consistently induced late, such as VSPs encoded by F-55 and H2339. The remaining wound-induced genes showed an intermediate pattern that is similar to proteinaceaous defenses like TI and PPO.

Interestingly, the leaf age differentially affects the induction of Pop3 (F-13) and Pop3-like (F-3.4.11). Pop3 (F-13) showed a leaf age bias for the younger leaves 3-5 while the Pop3-like (F-3.4.11) demonstrated an expression bias for the older leaves 9-11. This has been consistently demonstrated in preliminary Northern blots. The differential induction by leaf age of these two related genes is interesting and presents an opportunity for further research. Promoter analysis of these genes may

reveal if or how wound-induction is influenced by the developmental stage of leaf development.

F-1.6.7 and F-2.6.6 were identified in the screen but were not confirmed as being wound-induced on the Northern blots. Like the other constitutively expressed genes, they tended to be more highly expressed in leaves 3-4. Since the SSH library was constructed from these young leaves, small differences in developmental ages in plants could have given rise to their apparent induction quotient (see Summary and Discussion in Chapter 5). Interestingly, the basic endochitinase (H1756) is not wound-inducible, unlike all the poplar acidic endochitinases, i.e. *win6* and *win8* (H2339).

An examination of the Northern blots in Figure 4-3 reveals that all of the wound-induced genes, both novel and previously identified, appear to be induced by 12 h except the poplar VSPs (F-55 and H2339). Both VSP-win4.5 (H2339) and F-55 (VSP-PNI288-like) appear to be induced abruptly and strongly induced by 36 h. A similar trend occurred with the systemic time-course. By 12 h the VSPs were weakly induced at best, but by 24 h there appears to be an abrupt induction. The same pattern was apparent in preliminary blots (data not shown), and so it may indicate an underlying biological process such as a role in nutrient mobilization during defense. The VSPs are believed to be involved in the transient storage of nutrients as tissues undergo changes in sink status or nutrient demand, therefore, the initial induction may be due wounding but the later strong induction may be made be in response to the change in metabolic demands as the defensive preparations progress (see Chapter 6).

In summary, of the 17 unique sequences selected after the differential screening of the macroarrays, eight were confirmed to be wound induced. Of these eight, six were novel genes not previously published in poplar. All of the genes that were induced by mechanical-wounding were also induced systemically. Acid phosphatase and apyrase appear to be induced by 1.5 h and appear to be induced as strongly as the well known defense gene TI-*win3.12* (F-51). Pop3 (F-13) and Pop3-like (F-3.4.11) are induced early and demonstrate differential induction based on leaf age. The poplar VSPs F-55 and VSP-*win4.5* (H2339) demonstrate a late (and abrupt) wound-induction that may be in response to factors other than wounding.

CHAPTER 5

GENE EXPRESSION AFTER DIFFERENT TYPES OF WOUNDING, ELICITOR TREATMENTS AND IN UNWOUNDED POPLAR TISSUES

A. INTRODUCTION

Events that wound leaves can result in different forms of damage. The resulting damage from a crushing event is quite different than a piercing wound. Similarly, excision of poplar leaf material by mechanical means (razor) is different than insect herbivory and results in minor wound-induction (data not shown). Some plants are able to differentiate between some different types of damage and, in turn, induce different sets of defense genes (Mattiacci *et al.*, 1995; Alborn *et al.*, 1997; Korth and Dixon, 1997; Pohnert *et al.*, 1999; Halitschke *et al.*, 2001). It is also possible that the genes not showing wound-induction systemically or by mechanical-wounding may be responsive to some unknown FTC elicitor. In order to test poplar for the ability to distinguish between different types of wounding events, an experiment was performed to directly compare FTC-herbivory to two forms of mechanical damage. To do this, mechanical damage was performed by crushing leaves with a hemostat (surgical pliers) or by using a hand held hole-punch, and by matching the FTC damage directly.

Genes that are part of a defensive wound-response and not a secondary response to changes in metabolic demands should be responsive to chemical wound-elicitors such as MeJa. It is known that in poplar the application of exogenous MeJa, a likely intermediate in the wound-signal cascade, induces the defense genes TI and PPO (Haruta *et al.*, 2001 ; Constabel *et al.*, 2000). To test the novel genes for induction through the octadecanoid pathway, MeJa spray was applied to some poplar trees. In addition, to see if any of these genes were involved in systemic acquired

resistance (SAR), SA spray treatments were also performed. SA is known to be required for and induce many genes during SAR (Molina *et al.*, 1999).

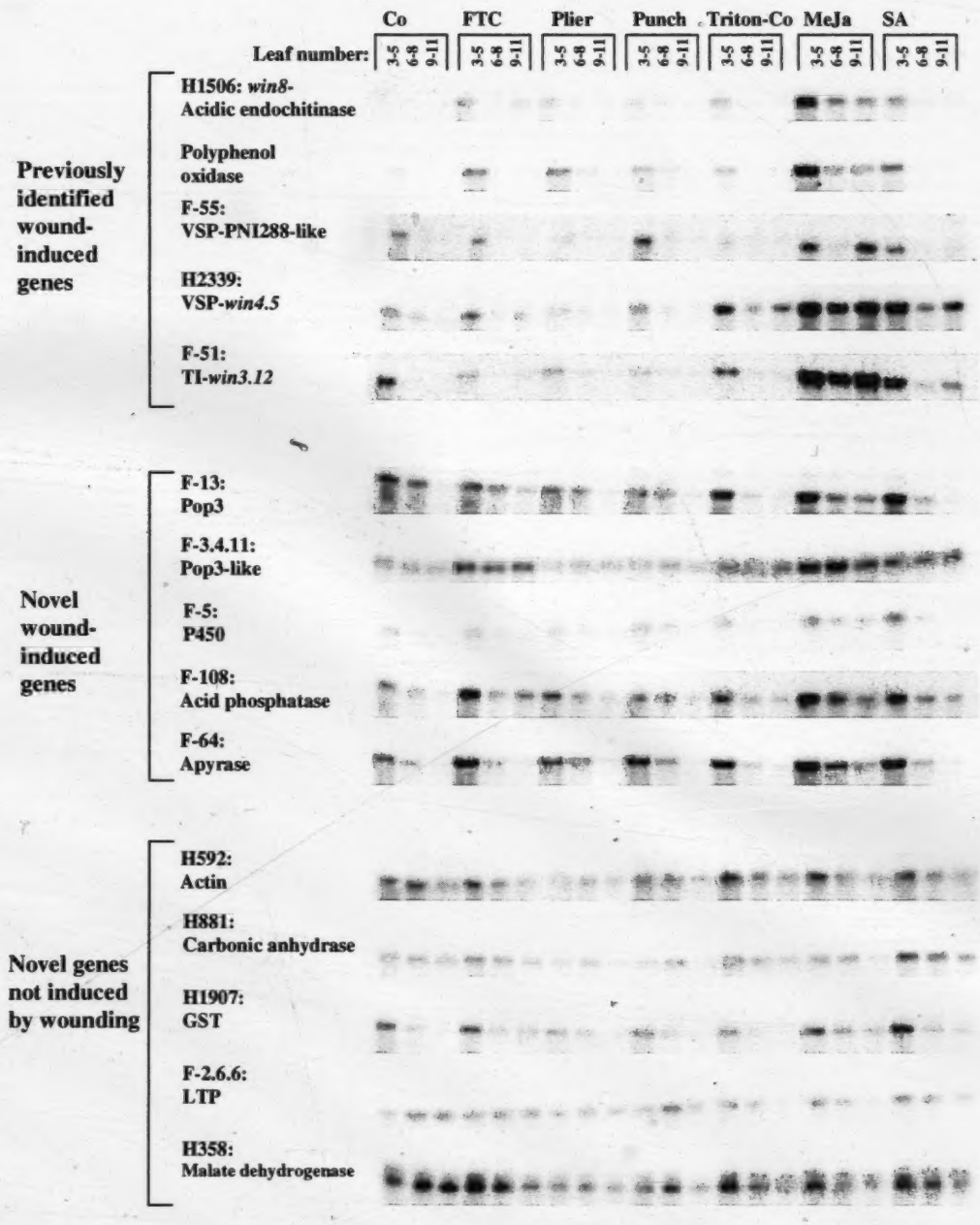
The defense signaling intermediate MeJa is also involved in tissue development which provides a link between defense and development. Therefore, defensive proteins such as protease inhibitors may be expressed during the development of storage tissues such as seeds and tubers. Some VSPs that respond to sink status are wound-inducible and may perform a role in the temporary storage of nitrogen and carbon during the metabolic adjustment to demands imposed by defense activation (Franceschi and Grimes, 1991; Mason and Mullet, 1990; Mason *et. al.*, 1992; Staswick *et al.*, 1991). These links between defense and development suggest that some defense genes could be expected to be expressed developmentally in poplar. In order to test this, a variety of poplar tissues were examined for gene expression by Northern blot analysis.

B. RESULTS

The results of the experiments comparing different induction treatments are shown on Figure 5-1. Again, samples are grouped by leaf age. In general, this experiment confirmed that the genes previously identified as wound-inducible are upregulated by other types of damage as well, and that young leaves are generally more responsive. It also confirmed that the genes that did not show wound-induction in the previous time-courses did not show wound-induction by FTC, plier, hole-punch, MeJa or SA. For the wound-induced genes, it is clear that MeJa induced all of the wound-induced genes, despite the higher background due to Triton x-100. The

Figure 5-1: Comparative Wounding and Elicitor Spray Treatments

The comparative wounding and elicitor time course was performed by direct wounding or elicitor treatment to the sampled leaves. For comparative wounding, FTC damage was mimicked in time and space on other trees by plier (hemostat) and holepunch damage. The elicitors or Triton control were sprayed on the entire tree. Each leaf tissue set consisted of three adjacent leaves from positions 3-5, 6-9, 10-12. Single trees were sampled 24 h after treatment. Similar FTC experiments have been performed two times using different leaf sampling patterns which yielded similar results.


Co
FTC
Plier
Punch
Triton-Co
MeJa
SA
Leaf number:
3-5
6-8
9-11
Previously identified wound-induced genes
H1506: win8-
Acidic endochitinase
Polyphenol oxidase
F-55:
VSP-PNI288-like
H2339:
VSP-win4.5
F-51:
TI-win3.12
Novel wound-induced genes
F-13:
Pop3
F-3.4.11:
Pop3-like
F-5:
P450
F-108:
Acid phosphatase
F-64:
Apyrase
Novel genes not induced by wounding
H592:
Actin
H881:
Carbonic anhydrase
H1907:
GST
F-2.6.6:
LTP
H358:
Malate dehydrogenase

high background expression is, for the most part, limited to samples from leaf positions 3-5. If samples 6-8 and 9-11 are examined carefully, the induction by MeJa becomes more apparent. TI (F-51) is the most obviously MeJa induced known-defense gene. This gene was not, however, induced by wounding and herbivory as expected. The lack of induction is an artifact of this particular experiment; previous experiments have consistently demonstrated induction by plier wounding and FTC-herbivory. Acid phosphatase (F-108), Pop3 (F-13) and especially Pop3-like (F-3.4.11) appeared to be more strongly induced by FTC-herbivory than by either of the mechanical wounding treatments. If these results are confirmed, than these three genes are responding to factors specific to FTC- herbivory and not just damage.

The SA treatments have not been verified by other experiments and so these results should be considered preliminary. Compare to the Triton control, SA appeared to induce TI (F-55) in leaves 9-11 and VSP-*win4.5* (H2339) in leaves 3-5. It may also have induced VSP-PNI288 at positions 9-11 since all the other samples except the MeJa treatment do not have expression. The other known wound-induced genes, PPO (PtdPPO) and acidic endochitinase (H1506), were not induced by SA.

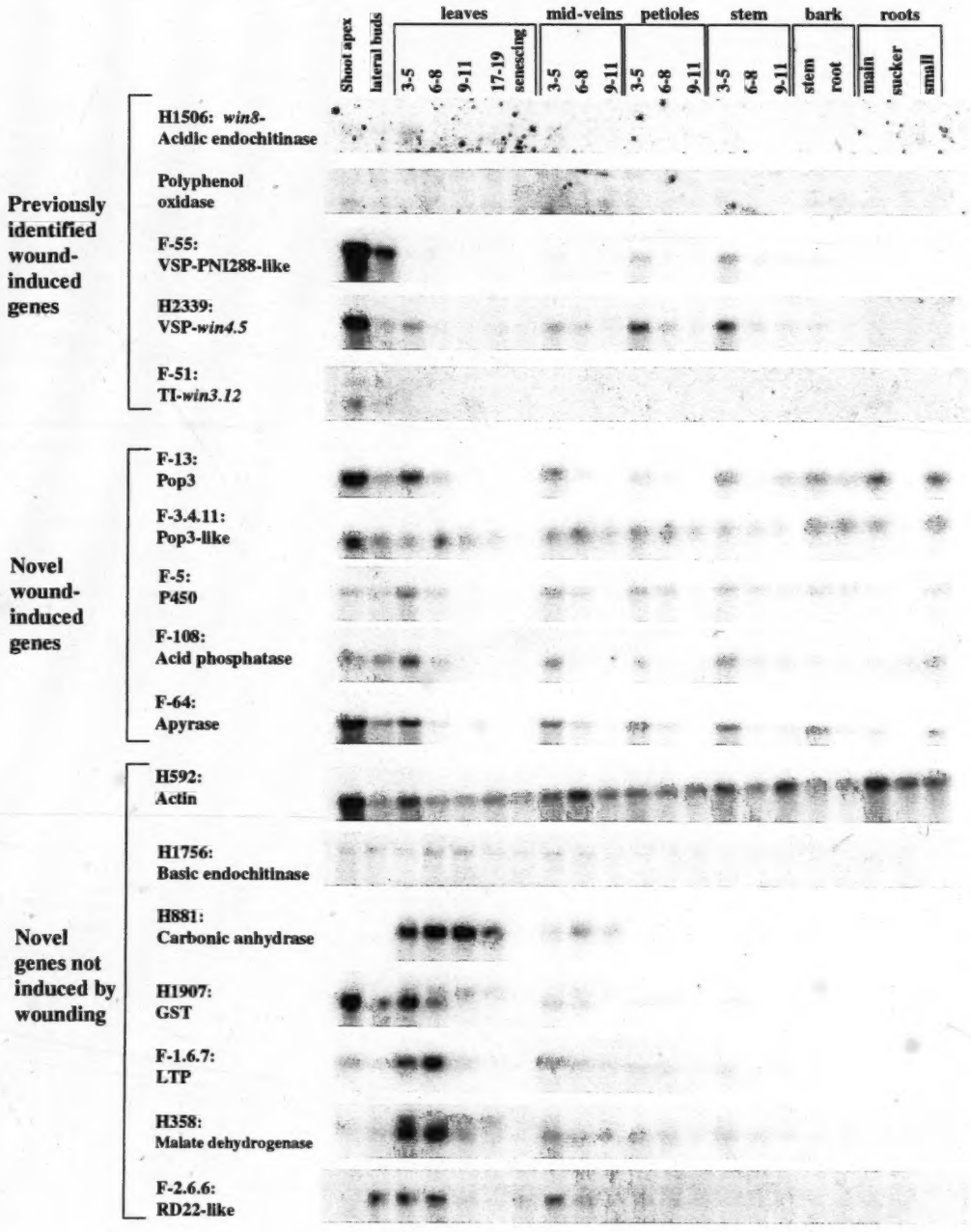
Sample variation was problematic in the experiment to compare mechanical-wounding to FTC-herbivory. Due to the unpredictable pattern of FTC-herbivory, each experiment is represented by a single tree. The experimental variation created problems for interpreting the wound-induction, primarily because of higher than expected backgrounds. The genes used as markers for wound-induction, TI and PPO, show poor induction. However, a few notable results are mentioned. Despite the high background seen in the young leaves, MeJa induced all of the novel wound-induced

genes. The induction is more evident in leaves 6-8 and 9-11 where the background expression is lower. This means that the novel wound-induced genes are likely regulated via the octadecanoid pathway. A second interesting result is that three novel genes may be responding specifically to FTC-herbivory. In this experiment, acid phosphatase (F-108) and apyrase (F-64) were both more strongly induced by FTC-herbivory at positions 3-5 than by pliers or hole-punch. Pop3-like (F-3.4.11) is more strongly induced in all leaf positions by FTC than by pliers or hole-punch. Replication of the experiment would clarify these results.

Examination of the poplar tissue survey in Figure 5-2 revealed a few interesting trends. The VSPs show strong developmental expression in the shoot apex and buds as expected (Lawrence *et al.*, 2001; Lawrence *et al.*, 1997) while the known defense genes TI, PPO and *win8* acidic endochitinase (H1506) revealed very little expression. Although, PPO and TI were expressed in the developing leaves the expression was much lower than expected (Haruta *et al.*, 2001; Constabel *et al.*, 2000). It is interesting to note that the previously identified wound-induced genes showed little constitutive expression in the other poplar tissues compared to the novel wound-induced genes. The acid phosphatase (F-108) appears to have constitutive expression intermediate between what is seen in the VSPs and the novel wound-induced genes, that is, it has a stronger "age gradient" than the other novel wound-induced genes. The novel wound-induced genes show nearly identical patterns of constitutive expression and, as noted previously, they all showed induction by MeJa. It is known that MeJa plays a role in the development of leaves and other tissues in many plants and is likely performing the same role in poplar. MeJa is one possible

Figure 5-2: Gene Expression in Unwounded Poplar Tissues

Gene expression in unwounded poplar tissues: demonstrating the expression of defense genes during development. Poplar leaves were sequentially labeled and sampled in sets as denoted on the figure. Tissue sets of the midveins, petioles and stem sections were numbered according to their associated leaf positions. The shoot apex included the unfolded leaves. The lateral buds were excised from next to the petioles from leaf positions 6-14. Three senescent leaves were pooled from three different H11-11 clones. The bark from the stem and main root (main stem below ground) was peeled off with a razor. The big roots were approximately 5 mm in diameter and the small roots were less than 2 mm. The sucker roots grew near the surface at the outer edge of the pot.


Shoot apex
lateral buds
leaves
3-5
6-8
9-11
17-19
senescing
mid-veins
3-5
6-8
9-11
petioles
3-5
6-8
9-11
stem
3-5
6-8
9-11
bark
stem
root
roots
main
sucker
small
Previously identified wound-induced genes
H1506: win8- Acidic endochitinase
Polyphenol oxidase
F-55: VSP-PNI288-like
H2339: VSP-win4.5
F-51: TI-win3.12
Novel wound-induced genes
F-13: Pop3
F-3.4.11: Pop3-like
F-5: P450
F-108: Acid phosphatase
F-64: Apyrase
Novel genes not induced by wounding
H592: Actin
H1756: Basic endochitinase
H881: Carbonic anhydrase
H1907: GST
F-1.6.7: LTP
H358: Malate dehydrogenase
F-2.6.6: RD22-like

direct link between the common expression of these genes constitutively in poplar tissues and after wound-induction.

C. SUMMARY AND DISCUSSION

An examination of the constitutive expression of the novel genes that did not show wound-induction reveals why they were selected during the differential screening of the macroarrays as false positives. With the exception of actin and H1756, the remaining novel genes all showed relatively high constitutive expression in leaves 3-5. If there were even small differences in leaf development between the control and induced samples used to make probes during the macroarray screening process, it would result in the identification of genes showing strong developmentally regulated expression. It could be possible that some of the "induced" genes from the macroarray screen are the result of the control leaf sample being more mature than the wounded samples. This would result in the genes involved in leaf development to be erroneously measured as induced. Since developing tissue was used to provide control and induced samples to make the SSH library, the problem of selecting false positives would be further amplified if these genes were enriched during the subtractive process as they would then make up a larger portion of the library.

There are a several reasons that might explain the constitutive expression of wound-induced genes during development. Expression seemed to be primarily in developing tissues such as the apical meristem, buds and developing leaves and in some cases the small roots. In addition, expression of many wound-induced genes could also be seen in the bark samples. These tissues are all nutrient sinks. Most of

these tissues are susceptible to insect or pathogen attack since they have not yet developed the physical barriers characteristic of older tissues, therefore, it is appropriate to express defense genes. Bark tissues in poplar serve to store nutrients particularly nitrogen in the form of VSPs. The poplars were grown in the environmental chambers and received a much higher supply of nutrients than wild populations. High nutrient status, nitrogen in particular, can induce VSPs and may explain the expression of genes in developmentally mature bark tissues (Coleman *et al.*, 1994; Lawrence *et al.*, 1997). The effect of nitrogen status on other wound-induced genes has not been well characterized. The transport of the poplar wound-signal is known to be similar to assimilate transport (Davis *et al.*, 1991) and preliminary experiments have revealed that poor light conditions result in a poor wound-induction of TI in poplar. Therefore the expression of the wound-induced genes may also reflect the sink status of developing tissues regarding fixed carbon. One additional thread that ties gene expression together through these different tissues is the presence of developing vasculature. Vascular tissue is believed to carry the wound-signal and is known to transport sucrose and fixed nitrogen. I would predict that *in situ* hybridization with the cDNA probes of the genes showing a strong link to leaf development, or antibodies to the expressed proteins, would demonstrate that the expression is localized primarily in the vascular tissues rather than the leaf lamina.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

A. POTENTIAL ROLES OF THE NOVEL *POPULUS* GENES DURING DEFENSE

The previous chapters of this thesis have described the identification and characterization of a number of wound-induced genes in poplar. Based on patterns of expression, it is hypothesized that they contribute to the defense in poplar at some level. Their contribution to defense is not immediately obvious based on the predicted functions of the gene products. In this chapter, the biochemical and physiological roles of these genes is described further, and some potential functions in plant defense explored. These could form the basis of future projects. I have chosen to discuss the novel-induced genes which I consider the most interesting and probably relevant for defense.

A-1 Apyrase: F-64

F-64 and F-101 have similarity to legume apyrases: lectin nucleotide phosphohydrolases (LNP). LNPs catalyze the hydrolysis of phosphoanhydride bonds of nucleoside di- and triphosphates and are involved in early nodulation events, and possibly the recognition of specific bacterial symbionts (Etzler *et al.*, 1999). The early time of induction for F-64 in poplar is similar to LNPs since a *Medicago truncatula* LNP *Mtapy1* shows mRNA transcript accumulation 3 h after inoculation with *Sinorhizobium meliloti* (Cohn *et al.*, 2001). The induction of F-64 in poplar leaves was not specific to FTC herbivory but appeared to be a general wound response. Since LNPs for *Dolichos* are carbohydrate-binding lectins, F-64 may be involved in the recognition of elicitors from damaged plant tissue, bacterial or fungal elicitors, or

herbivore elicitors. Its role in early defense may be in sensing damage, pathogens or herbivores or act to modify the suite of defense genes induced. LNP shows increased apyrase activity after binding to a nodulation factor from *Sinorhizobium melitoti* (Etzler *et al.*, 1999). In legumes, LNP homologs demonstrate a functional role in nodulation since nodulation in *Dolichos biflorus* and soybean is prevented by antisera raised against their respective LNPs (Cohn *et al.*, 2001; Day *et al.*, 2001; Etzler *et al.*, 1999). Alternately, the apyrase-like genes may be important for changes in nutrient status of mobilization which could occur during the wound response (see below).

A-2 P450: F-5

P450s are a class of heme containing oxygenases which typically carry out hydroxylation reactions in plants, especially in secondary metabolism and are involved in a multitude of reactions and metabolic pathways, including the biosynthesis of major plant defense compounds such as phenylpropanoids (Winkel-Shirley, 2001). Many genes involved in this pathway are known to be strongly induced in aspen by wounding and insect herbivory (Peters and Constabel, 2002). The expression of the P450 in poplar is consistent with a role in defense. It is induced by direct and systemic wounding as well as MeJa treatments. F-5 has strong sequence similarity to an elicitor induced 5-epi-aristolochene-1,3-dihydroxylase from tobacco involved in terpenoid metabolism (Ralston *et al.*, 2001). In *Nicotiana tabacum*, the main antimicrobial agent capsidiol is produced via the isoprenoid pathway in which 5-epi-aristolochene-1,3-dihydroxylase is involved. It is possible that F-5 has a similar function, although the actual substrate cannot be predicted by its primary sequence.

Since F-5 is a partial clone it is also possible that the complete protein could show stronger sequence similarity to P450s involved in other pathways. It is likely that F-5 is involved in the synthesis of defensive secondary metabolites, but more research is required to determine its role.

A-3 Pop3-like Sequences: F-13 and F-3.4.11

The expression of the Pop3 and Pop3-like sequences are consistent with a role in defense, since they are both inducible by direct and systemic wounding, as well as MeJa treatment. Interestingly, they show an opposite leaf age expression bias, with F-13 more highly induced in the younger leaves and F-3.4.11 in the older leaves. Drought, hot and cold temperature, and abscisic acid application induce Pop3 (also called BspA and SP1) (Pelah *et al.*, 1995; Wang *et al.*, 2002).

F-13 and F-3.4.11 are part of a gene family in poplar, which I will name Pop3-like for convenience, that consists of F-13, F-3.4.11, and at least one related poplar EST sequence (B1071535) with a C-terminal extension. In *Arabidopsis*, the Pop3-like gene family consists of four related sequences. Two sequences are of similar length to F-13 and F-3.4.11 and two longer related sequences that have C-terminal extensions of about 110 amino acids (data not shown). Pop3-like genes are conserved across the plant kingdom with over 280 plant ESTs in the database. There are Pop3-related sequences in dicots, monocots, gymnosperms, bryophytes and even bacteria, which suggest that they perform a significant and similar function. The most plant-like bacterial Pop3-like sequence is found in *Vibrio cholerae*. Using PSI/PHI-BLAST analysis it was possible to retrieve several other bacterial Pop3-like sequences.

PSI/PHI-BLAST analysis revealed that a bacterial sequence *Hydrogenophilus thermoluteous* is an in-frame fusion of a Pop3-like sequence and a fructose 1,6-bisphophate aldolase (BAA75221). Thus, the Pop3-like sequence of *H. thermoluteous* is not only expressed by the same promoter, it is also physically linked to fructose 1,6 bisphosphate aldolase. This would suggest that Pop3-like sequences may function in the same metabolic pathway as this enzyme, or even share an enzymatic substrate or product. Alternately, Pop3-like sequences may be protein modules that act as stabilizers or chaperones for different proteins. BspA is heat inducible and capable of stabilizing proteins during heat treatments (Wang *et al.*, 2002), and thus the fused Pop3-like sequence may be acting as a heat shock chaperone to the fructose 1,6 bisphosphate aldolase in the thermophilic *H. thermoluteous*. The nature of BspA's heat stability is intriguing since Pop3-like sequences do not have conserved cysteines with which to make disulfide bridges. It is also possible that the longer Pop3-like sequences with the C-terminal extensions in *Arabidopsis* and poplar also are fusions of Pop3-like modules that serve a chaperone stabilizing function. Consistent with the hypothesis that Pop3-like sequences are chaperones, BspA forms complexes with 16 subunits and can protect enzymes from protease digestion and serve a defensive function in this way (Wang *et al.*, 2002).

A-4 Acid Phosphatase (VSPα–like): F-108

Poplar acid phosphatase F-108 (VSPα-like) is also strongly induced by both direct damage and systemic wounding. The acid phosphatase is a homolog to legume acid phosphatases such as VSPα and VSPβ and is a novel sequence to the poplar

system. VSPα is a storage protein that responds to nitrogen status, drought, and MeJa, and accumulates in leaves after depodding (Franceschi and Grimes, 1991; Mason and Mullet, 1990; Mason *et al.*, 1992; and Staswick *et al.*, 1991). VSPα was later found to have weak acid phosphatase activity with low substrate specificity (DeWald *et al.*, 1992), so it may play a different function in defense. Soybean VSP antisense plants do not demonstrate any effect on plant growth compared to wild-type in the field (Staswick *et al.*, 2001). There is some evidence that would suggest that these proteins function primarily as storage proteins. Protein sequences are strongly conserved between VSPα acid phosphatase homologs in soybean, *Arabidopsis*, and poplar (data not shown), suggesting that perhaps they share an important function and substrate. There is evidence that the F-108 homolog, *AthVSP*, in *Arabidopsis* plays a crucial role in developing reproductive structures. This was surmised because male sterility in *Arabidopsis* flowers in a coronatine/MeJa insensitive mutant appear to be the result of a missing *AthVSP* correlated 29-31 kDa protein normally abundant in these tissues (Benedetti *et al.*, 1995; Berger *et al.*, 1995; Feys *et al.*, 1994). At first glance this would appear to contradict the results obtained from the soybean antisense plants, however, only VSPα was down-regulated and it is possible that VSPα and VSPβ perform a redundant role. Therefore, sterile flowers in *Arabidopsis*, similar wound and tissue expression patterns in different plants suggest that these proteins function as more than simple storage reserves.

A-5 Vegetative Storage Proteins

A-5.1 F-55 and H2339

Sequencing revealed that F-55 encodes a VSP nearly identical to the VSP, PNI288, described in apical buds (Lawrence *et al.*, 2001) and that the F-55 sequence contained the missing N-terminus of PNI288. F-55 and PNI288 are related to the well-characterized VSPs *win-4.5* (H2339) and bark storage proteins (BSPs) from poplar (Lawrence *et al.*, 2001). Therefore, there appear to be several wound-induced VSPs in poplar leaves. F-55 and VSP-*win4.5* (H2339) sequences have homology to phosphorylases of pfam01048; this is the first postulation of an enzymatic function. The low amino acid conservation of F-55 and VSP-*win4.5* to pfam01048, 24% and 27% similarity respectively, is bolstered by the fact that sequences in other plants related to F-55 and VSP-*win4.5* also have homology to pfam01048. Similar peptides predicted from *Arabidopsis* (AF-370278), *Medicago truncatula* (BF-635374), *Mesembryanthemum crystallinum* (BE035944), *Lycopersicon esculentum* (AW092074), and *Secale cereale* (BE587154) are a few of the many related EST sequences that also show homology to pfam01048. In poplar, the related BSP and VSP genes both belong to different clustered gene families that are linked within 5cM (Davis *et al.*, 1993). The sequence divergence between VSP members suggests that they are not under strong substrate binding constraints which may imply that their enzymatic activity is not important (see Future Work this Chapter). Another possibility is that they may have several potential substrates and that their divergence is being driven by selective pressures towards specific substrates presumably related to defense.

H2339 and F-55 transcripts are known to be abundant in the shoot apex (Figure 5-2). Immunolocalization was used to examine VSP in developing terminal buds reveals that the cell types that accumulate storage protein during terminal bud development are fundamentally different than the cell types that express WIN4 during growth (Lawrence *et al.*, 1997). As bud formation progressed, storage protein accumulated in the ground meristem beneath the apical dome and in cells adjacent to xylem. The localization of storage protein suggests that it is available for remobilization for shoot growth when bud dormancy is broken. In contrast, WIN4 is located in stipules and in developing leaves in actively growing shoot apices (Lawrence *et al.*, 1997) and may be acting as a buffer for temporary storage of nitrogen. Like soybean VSPα, H2339 and F-55 showed expression in developing leaves and associated tissues and especially the apical meristem. VSP-PNI288 and VSP-*win4.5* showed a late induction of about 18 h that may reflect that they are part of a sequential activation of defense genes or that they are acting as a storage reserve during protein turnover and nutrient mobilization later in defense.

A-5.2 VSPs: Defense and Development

Analysis of the constitutive expression of the wound-induced genes revealed a link between defense and development, which may be related to nutrient or sink-status of the tissue examined. For example, many of the wound-induced genes in leaves are also developmentally expressed in bark, a known storage tissue in poplar (Figure 5-2). This may have arisen due to the ecological niche that *Populus* fills. Most *Populus* species inhabit riparian habitats where they fulfill critical ecological roles in

the protection of riparian habitats and of the adjacent floodplains from bank erosion and sedimentation. Poplar physiology and recruitment is closely intertwined with the spatial and temporal dynamics of river systems. The seasonal fluctuations in river discharge resulting in occasional peak flows, erosion and accretion, and changing meanders over time are critical elements in the recruitment and establishment of riparian poplar (Braatne *et al.*, 1996; Mahoney and Rood, 1998). These seasonal variations in river flow result in riparian ecosystems receiving episodic inputs of nutrients during the deposition of sediment during flooding events which may explain why poplars tissues store high levels of nitrogen in the form of vegetative storage proteins for subsequent utilization (Coleman *et al.*, 1994; Lawrence *et al.*, 1997). Within the same tree, storage proteins and nitrogen levels fluctuate dynamically among organs in concordance with seasonal rhythms of active growth and dormancy. When nitrogen availability is high, *Populus* induce TIs (in addition to BSP and VSPs) demonstrating that the expression of a defense gene may be linked to nutrient status (Constabel, unpublished data).

Many of the same kinds of proteinaceous defenses expressed in poplar are also expressed in herbaceous plants in seeds and storage tissues. Indeed, several wound-inducible genes are expressed in developing seeds and storage tissues. Poplar chitinase-GUS constructs are expressed in poplar floral tissues (Clarke *et al.*, 1994), TI and a wound-inducible bark storage protein (BSP) are expressed in poplar seeds (Hollick and Gordon, 1995; Zhu and Coleman, 2001) indicating an overlap in defense and development. The link between defense and development may in part be due to the fact that jasmonates are involved in regulating several processes including

abscission, drought stress, and tendril coiling (Hartmond *et al.*, 2000; Hall and Horton 1994; Curtis, 1984; Richard *et al.*, 2000; Chao *et al.*, 1999; Forsthoefel *et al.*, 1998; Engelberth *et al.*, 2001; Blechert *et al.*, 1999; Weiler *et al.*, 1993). Alternately, these tissues may be constitutively protected, because of their importance to the tree's survival.

This research has revealed that there may be some similarities between wound-induced defense in leaves and young developing tissues. The connection appears to be the presence of developing vasculature in these tissues. The link between vasculature and the expression of defense genes may lie with the expression of allene oxide synthase (AOS), a key enzyme in jasmonate synthesis. In situ hybridizations using barley seedlings revealed the preferential occurrence of AOS mRNA in parenchymatic cells surrounding the vascular bundles of the scutellar nodule of the developing seedlings (Maucher *et al.*, 2000). In soybean, the highest jasmonate levels were observed in pericarp vascular bundles (Lopez *et al.*, 1987). In soybean, immunolocalization of VSP (acid phosphatase) protein, *in situ* hybridization with an RNA probe to VSP mRNA, and in situ localization of acid phosphatase activity is localized to the paraveinal mesophyll cells located adjacent to the vascular bundles (Klauer *et al.*, 1996; Staswick *et al.*, 1994; Huang *et al.*, 1991). In poplar, VSP-*win4.5* is localized to cells peripheral to vascular bundles in poplar leaves (Lawrence *et al.*, 1997). The link between defense and development may be the cell types associated with the vascular bundles. Vascular bundles and elements are ubiquitous in plant tissues, and it is likely that vascular tissues play a role in disseminating systemic signals to adjacent cells and tissues both in the bark and in

leaves. These cells likely play a crucial role in octadecanoid signaling by being the (initial) source of the octadecanoids that would then disperse to other tissues during wounding and development as suggested by Orozco-Cardenas *et al.* (2001).

Poplar acid phosphatase (F-108) represents a homolog to the soybean VSPα, which has demonstrated acid phosphatase activity (see Chapter 3). The poplar VSP sequences VSP-*win4.5* (H2339), F-55 (PNI-288-like), and BSP (data not shown) have a phosphorylase domain. Perhaps it is only coincidence that these unrelated sequences have potential functions in phosphate metabolism. However, phosphate status is known to effect wound induction. High phosphate levels tend to suppress wound-induction whereas low phosphate levels tend to increase the wound-induction of wound-responsive genes in soybean (Sadka *et al.*, 1994; Berger *et al.*, 1995). However, in preliminary experiments, modifying the phosphate supply to greenhouse-grown poplar did not change the induction of TI and other defense proteins (Cory Miller and Peter Constabel, unpublished data).

A key question is if the enzymatic functions ascribed to the poplar VSPs F-55, *win4.5* (H2339) and F-108 acid phophatase are necessary for their defensive function. If they are simply VSPs responding to sink status and development it is likely that enzymatic activity is not essential for their storage roles. It is therefore possible that these genes act as VSPs and that their role in defense is secondary. Perhaps they are involved in nutrient mobilization or storage during the metabolic changes during the defense response. The late wound-induction seen for VSP-*win4.5* and F-55 would fit this pattern (Figures 4-2 and 4-3). Since an antisense knockout of VSPα has demonstrated that plant productivity is not compromised (Staswick *et al.*, 2001), the

function of VSP in soybean is still unclear. However, these plants may perform differently than wild-type under herbivore pressure where a deficiency in defense might become obvious. The connection of VSP expression to phosphate can be explained without invoking a direct defensive function. The addition of hexoses result in a decreased phosphate pool in cells as the sugars get phosphorylated (Mason *et al.*, 1992), and the phosphate pool may therefore be a measure of sink status for fixed carbon. It is possible that the poplar acid phosphatase and poplar VSP-*win4.5* (H2339) and F-55 have been recruited as storage proteins because they originally responded to decreased cellular phosphate pools as functional enzymes and now their enzymatic domains are vestigial. To pursue this question, it will be necessary to determine if their activities are also induced by wounding and whether their activity has a necessary function in the defense response.

B. FUTURE WORK

Although much new information has been gained through the bioinformatics and research on gene expression presented in this thesis, this new suite of wound-induced genes will continue to be a valuable research tool to analyze gene expression in the poplar model system. Some genes, such as LTP, appeared to be wound-induced during the macroarrays analysis but this was not confirmed during the subsequent Northern analysis of the wounding time-courses or the elicitor treatments. LTP may be induced by additional factors that were not replicated, such as the presence of microbial populations in the environmental chambers or transferred by different batches of FTC through regurgitant. During the final stages of preparing this thesis it

was confirmed that FTC regurgitant contains volicitin and appears to be capable of inducing TIs in poplar after a substantial dilution of 1/100 and will likely become a very useful tool for the further study of poplar-FTC interaction (Ian Major and Peter Constabel, unpublished data).

Other problems of this nature can be analyzed using this new suite of wound-induced genes in combination with elicitors derived from bacterial or fungal pathogens to test for differential induction. It is possible that some of these genes will respond differentially and thus become tools to dissect different aspects of the wound-signaling pathway.

A major finding of this work is the wound induction of genes primarily thought to function in nutrient mobilization. The expression of three genes involved in phosphate metabolism during defense is a novel finding. This thesis showed that apyrase (F-64), phosphorylase (F-55, H2339) and acid phosphatase (F-108) all appear to be involved in some aspect phosphate metabolism and defense. Since VSPs are also affected by nitrogen status, the link between nitrogen or phosphate metabolism and wound-induction can be further analyzed using these specific genes. It is possible that some of the other novel genes will also be regulated by nutrient status. In addition to examining responses to nutrient availability it would be interesting to test the poplar VSPs for phosphorylase activity to see if the phosphorylase domain is active or vestigal. This could be accomplished using bacterial expression vectors and artificial substrates.

The F-71 clone provides a unique opportunity to study a poplar gene of unknown function using the *Arabidopsis* model system. F-71 has strong homology to

a predicted peptide from a single copy gene in *Arabidopsis*. TBLASTX analysis revealed that there are nearly 200 plant ESTs in the database that shows very strong homology to the predicted F-71 peptide. A search of the NCBI database revealed that F-71 homologous peptides are predicted across and restricted to the plant kingdom and can be found in dicots, monocots, gymnosperms, and bryophytes. The F-71 ortholog in *Arabidopsis* (AAL11546) exists as a single copy and a T-DNA tagged knock-out (SALK_035046) is available, making it an excellent candidate for transgenic analysis.

Pop3 is boiling stable (Pelah *et al.*, 1995) and has chaperone-like properties (Wang *et al.*, 2002). It can confer protection to other enzymes during heat denaturation or proteolytic degradation (Wang *et al.*, 2002). Therefore, it is possible that in poplar, the F-13 and F-3.4.11 Pop3-like sequences act as part of the anti-nutritive defense by preventing the digestion of ingested protein. This could be tested on several levels. A bacterial expression vector could be constructed to express and purify these gene products. The purified proteins could then be tested to see if they are able to protect a heat-labile enzyme from heat denaturation. They could also be used to see if they can confer protection from enzymatic degradation of a commercial enzyme such as β-glucosidase. Since BspA is boiling stable, F-13 could easily be tested in an artificial diet to see if it inhibits the digestion of protein and results in an increase in bypass protein in insect frass and decreased insect performance. Pop3-like sequences could be tested to see if they confer protease or heat-denaturation protection as a protein module by creating N- or C-terminal fusion proteins to an enzyme such as β-glucosidase. If Pop3-like proteins perform a role in dehydration,

transgenic canola or *Arabidopsis* seeds could be germinated on agar plates containing salt to see if dehydration protection correlates to Pop3-like protein expression. In addition, there are two Pop3-like sequences in *Arabidopsis* that could potentially be down-regulated using RNAi if T-DNA tagged sequences do not become available. Plants with down-regulated or knock-out of Pop3-like genes should be more susceptible to drought (salt stress) or even insect herbivory.

The above is only some of the possibilities for future research. Many additional avenues for research in both poplar and other plant systems such as *Arabidopsis* are now available using the new suite of wound-induced genes presented in this thesis.

C. CONCLUSIONS

The results presented in this thesis have expanded the number of known wound-induced genes in the poplar system, as well as plant systems in general. One gene, F-3.4.11, may respond specifically to FTC herbivory (Figure 5-1) suggesting the presence of an elicitor. The newly identified wound-induced genes all respond to MeJa and are, at least in part, regulated through the octadecanoid pathway. The F-71 and F-13 (Pop3) and F-3.4.11 (Pop3-like) sequences are novel wound-induced sequences that code for proteins of unknown function that were previously unpublished in any plant system. The P450 is a novel sequence that codes for a protein of unknown function that, like several P450s, is likely involved in secondary metabolism. Several genes, including acid phosphatase, apyrase, and the poplar VSPs, code for proteins that are involved in phosphate metabolism. Both the acid

phosphatase and apyrase have never been cloned from *Populus* and are novel sequences to the poplar system. The VSPs (F-55 and H2339) were previously identified but this work discovered a phosphorylase domain within the primary sequence that may shed light on the function or origin of these VSPs. In addition, the F-55 sequence has extended the known predicted PNI288 sequence at the N-terminus. Several sequences that were not wound-induced were also analyzed, and some had expression patterns that demonstrated links to leaf development. These included the lipid transfer protein (F-1.6.7), glutathione S-transferase (H1907), and carbonic anhydrase (H881). These genes were not induced by the wounding or elicitor treatments used but may respond to additional stimuli related to wounding. In addition to the information directly generated by the research presented in this thesis, more knowledge will accrue in the poplar model system from related projects extending from this research.

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APPENDIX I

SEQUENCES

The nucleotide sequences presented here were assembled primarily from sequences from the SSH library and the EST project (Mary Christopher and Lynn Yip). Sequences from the NCBI and PoplarDB databases were used to aid in sequence editing, bridging gaps between SSH and the in-lab EST project, as well as extending sequences where possible. The peptide sequences were predicted using BCM sequence utilities (see Material and Methods). The positions of the predicted start and stop codons were compared to those found in the best protein database matches found (BLASTP). The predicted start codon (**ATG**) and associated methionine (**M**) is labeled in bold type and highlighted in green. The predicted stop codon is labeled in bold type and highlighted in red ( ) and is designated by a bold, red highlighted asterix ( ).

Acid Phosphatase: F-108

Nucleotide:

GCACGAGC**ATG**GCTTCTTTAATTTTGCAAATCTTTTTTCTTGCAGTGATT
TTAGCAACTTCCCAGGGCTCCCCTCTGAGCTATTACCCCCTCCGGATTCA
CCTTCTCAGGTCAAAATGGGGTTCCGGCGACCACCACATTCCTGGCATGT
CATGCCTGAGTTGGCGACTAGCTGTTGAAACAAACAATGTGATTGGATGG
AGTACAGTTCCTGAAGAGTGTGAAGATTACGTAGGGCACTACATGTTAGG
CAGCCAATACAGGGAAGACTCATCTGTCATCACTGATGAGGCTTTTGCCC
ATGCTAAAACCTTCAAGCTGGCTGGAGATGGAAAGGATATATGGGTCTTT
GACGTTGATGAAACTACTCTTTCTAATTTACCCTACTATGCCAAACATGG
ATTTGGGGCGGAGCCTTACAATTCCACGGCGTTCAACCAATGGGTTTTCA
CGGGCAAAGCCCTAGCATTGCCAGAGTCTCTGAAGCTGTACCGGAACTTA
TTGTCAATTGGGATCAAGGTTGTGTTCTTAACGGGAAGGACTGAAGACCA
AAGAGCAGTGACATCAAACAATCTGAAGAATGCTGGGTACCACATCTGGG
AGAAGCTGATACTCAAGTCATCATCTTATTCAGGGAAGACAGCGGTGTTT
TATAAATCAAGCGAAAGGGCAAAGCTTGAGAAGAAAGGATACAGAATCAT
CGGGAACATTGGTGATCAGTGGAGTGATCTCCTGGGGACTAGTGTCGGCA
ATCGGACATTCAAGCTACCTGATCCAATGTATTACATCAGTTGAATCCTC
GGATTCTTAGCCTCTAGCTTGGTTAACTTGTATGATTCAGAT[redacted]TTGAT
GTTGTTAACAAAACC

Predicted Peptide:

MASLILQIFFLAVILATSQGSPLSYYPLRIHLLRSKWGSGDHHIPGMSCL
SWRLAVETNNVIGWSTVPEECEDYVGHYMLGSQYREDSSVITDEAFAHAK
TFKLAGDGKDIWVFDVDETTLSNLPYYAKHGFGAEPYNSTAFNQWVFTGK
ALALPESLKLYRNLLSIGIKVVFLTGRTEDQRAVTSNNLKNAGYHIWEKL
ILKSSSYSGKTAVFYKSSERAKLEKKGYRIIGNIGDQWSDLLGTSVGNRT
FKLPDPMYYIS[redacted]

Apyrase: F-64

Nucleotide:

GGCCGAGGTGCACAGCTAGCGCTGCAGATGTTCAAGAAGGCAGCTAAGCG
TGCTTGCGAAACAAGATTTGAGGATGCAAGCTCAAGATTTCCAAATGCGC
TGGAGGAAGACCTGCCATTCTTGTGTATGGATTTTACCTATGAGTATACT
TTGCTTGTCGATGGATTTGGTCTTCATCCCCAGAAAAAATTTTCAGTGGA
GGAGAAAGTTAAATACAAGAATTCACTTATGGAAGCTGCGTGGCCTCTTG
GTAGTGCTATAGAGGCGGTGTCAACC[redacted]CAG

Predicted Peptide (frame +1):

GRGAQLALQMFKKAAKRACETRFEDASSRFPNALEEDLPFLCMDFTYEYT
LLVDGFGLHPQKKFSVEEKVKYKNSLMEAAWPLGSAIEAVST[redacted]Q

Apyrase: F-101

Nucleotide:

ACTATAGTGTGGATGGCAATAAACTACATGGTAGGAAATCTGGAGAAGCC
GTATTCAGAAACAGCAGCAGTAATTGATCAAGGCGGTGGATCTGTTCAAA
TGGCATATGCCATTTCCAGGGAGAACGCTGAAAAGGCACCAACAGTAGCT
GATGGAGAGGATCCATACGTGGAGAAATTCCTTCTTAGAGGAGCCGAATA
TTACGTTTATGTTCACAGTTACTTGAGCTATGGATTATTGGCGTCTCGGG
CAGAAATACTGAAGGTTTCAAGAAACTCCAGCAACGAGTGCGTAGCCACC
GGTTATAATGGTGTTTACAAATACGGAGGGAAGGAATACAAAGCATCATC
TCCTCCTACCGGTACANGCTCCAAAANNTGCAGAACACTAGTTCTNAAAG
CTCNNCAAAGTNAATGCCCATGTAACTATGTGATGCACACCGGTGGAGTA
TGGAATGGCGGCAGCGANACGGGCAAAAC

Predicted Peptide (frame +1):

TIVWMAINYMVGNLEKPYSETAAVIDQGGGSVQMAYAISRENAEKAPTVA
DGEDPYVEKFLLRGAEYYVYVHSYLSYGLLASRAEILKVSRNSSNECVAT
GYNGVYKYGGKEYKASSPPTGTXSKXCRTLVLKAXQSXCPCNYVMHTGGV
WNGGSXTGK

Carbonic anhydrase: H881

Nucleotide:

ATGTCGACTGCTTCGATTAACAGCTGGTGTCTCACCTCTGTCTCTGCCTC
TAAGAGATCACTACCCGCATTACGTACTTCAGTCTTTGCTAGCCTCAACT
CCTCTGTTTCTCCTCCTACCCTTATCAGAAACCAGCCTGTTTACGCAGCC
CCTGCTCCTATTCTCTATCCACCTCGGAGAGGCGAAGAAATGGGAAAGGA
CTACAACGAGGCCATTGAATCTCTCAAGAAACTCCTCAGTGAGAAGGATG
AGCTGAAAACTGTAGCAGCTGCGAAAGTGGAGCAGATAACAGCTGAATTA
CAAACCGCCTCATCTTCTGACCCCAAGGCATTCGATCCTGTTGAGAAGAT
TAAATCCGGCTTCATTCACTTCAAGAAGGAGAAATATGACAAGAATCCGG
GACTGTACTCCGAGCTTGCCAAAGGCCAAAGCCCCAAGTTTATGGTGTTT
GCATGCTCGGATTCCCGGGTTTGCCCGTCCCATGTTCTTGATTTCCAACC
AGGGGAAGCTTTAGTGCTCCGCAATGTTGCGAATATGGTCCCGCCATACG
ATCAGACTAAGTACGCTGGAGTTGGGGCAGCAATAGAGTATGCAGTTTTG
CATCTGAAGGTGGAATACATTGTGGTCATAGGACACAGCGCCTGTGGTGG
AATGAAGGGCCTCATGTCCTTCCCTTATGATGGAACAACATCAACTGATT
TCATAGAAGACTGGGTCAAAGTCTGCTACAATGCCAAGACCAAGATTTTA
GCAGAACATGCCAACTCACCTTTCCCAGACATGTGTACACAATGTGAAAA
GGAGGCAGTGAACGTGTCCATCGGACACTTGCTCACCTACCCGTTTGTGA
GAGATGGCTTGGTGAACAAAACTCTAGGACTGAAGGGTGGTTATTATGAT
TTTGTCAAAGGCAGTTTTGAGCTCTGGGGGCTTGAGTACAGCCTCTCCCC
CTCTCTCTCCGTA[redacted]ACATCATCCAAATATCAGTGACCCTATCTTGGTT
CTTAACTTTCTTTCCCAGATGTACTTTGTAGATGCTTAAATGAGCTTTAT
TTTCGATCAAGATTAATGAACATATGAAATGGAGATTACCAGCATCTCCT
TCTCTAATAAATGCAGCAGCAGCACTCTATTATA

Predicted Peptide (partial):

MSTASINSWCLTSVSASKRSLPALRTSVFASLNSSVSPPTLIRNQPVYAA
PAPILYPPRRGEEMGKDYNEAIESLKKLLSEKDELKTVAAAKVEQITAEL
QTASSSDPKAFDPVEKIKSGFIHFKKEKYDKNPGLYSELAKGQSPKFMVF
ACSDSRVCPSHVLDFQPGEALVLRNVANMVPPYDQTKYAGVGAAIEYAVL
HLKVEYIVVIGHSACGGMKGLMSFPYDGTTSTDFIEDWVKVCYNAKTKIL
AEHANSPFPDMCTQCEKEAVNVSIGHLLTYPFVRDGLVNKTLGLKGGYYD
FVKGSFELWGLEYSLSPSLSV[redacted]TSSKYQ*PYLGS*LSFPDVLCRCLNELY
FRSRLMNI*NGDYQHLLL**MQQQHSII

Endochitinase: H1756

Nucleotide:

CACTTCCAACAAAGCTCACACAAGCTCTTCGACACGTACGCAGGCATAGC
CAAGATGGAGGCCAA**ATG**GTGTTTTCTTGTGACAATGGCGTTGTTAGTAA
TGTTAGTGGTAGTAGTAAATGGAGATGAATCATCTCAAGTAAAGACAGTG
GTGAAGATAGTGAAGGGAAAGAAGGTGTGCGACAAGGGGTGGGAATGCAA
AGGCTTGTCTGCCTATTGCTGCAACCAAACCATTTCTGATTTTTTCCAGA
CCTACCAGTTCGAAAACTTGTTTTCGAAACGTAACACTCCTGTGGCTCAT
GCTTCGGGATTTTGGGATTACCATTCTTTTATCACTGCAGCTGCAGAATA
CCAGCCTCATGGATTTGGTACCGCCGGAGGGAAACTTACGGGGCAGAAGG
AAGTTGCTGCTTTCCTTGGGCATGTTGGAAGCAAAACCTCATGTGGTTAT
GGAGTGGCCACTGGAGGACCACTGGCATGGGGTTTGTGCTACAACAAGGA
AATGAGTCCCAGCAAGACATACTGCGATGATTACTACAAGTACACCTATC
CTTGCACTCCCGGAGTTTCGTATCACGGCAGGGGTGCCCTTCCTCTTTAC
TGGAACTACAACTATGGCAAAACTGGGGAAGCCCTGAAGACTGATTTGCT
GAACCATCCAGAATACCTCGAAAACAATGCTACACTAGCTTTCCAGGCTG
CTATTTGGAAGTGGATGACACCAGAAAAGAAGCATCTCCCTTCAGCACAC
GATGTATTTGTTGGCAAATGGAAACCTACCAAGAATGACACTTTGGCCAA
GAGGGTACCTGGATTTGGCACCACCATGAATGTTCTGTATGGGGATCAAG
TTTGCGGCAAAGGCGATGATGAGTCGATGAACAACATTGTCTCTCATTAC
CTTTATTATCTCGACCTAATGGGTGTTGGCAGAGAGGAAGCCGGGTCTCA
TGAATGTGCTAGCTGTGCTGAGCAACTACCTTTCAACCAAGCCTCTGCCT
CTGCATCATCTACTAGAACTCGTGAG[redacted]TCTGTCATGTGACGTCTCTAC
TATGA

Predicted Peptide:

HFQQSSHKLFDTYAGIAK**M**EAKWCFLVTMALLVMLVVVVNGDESSQVKTV
VKIVKGKKVCDKGWECKGLSAYCCNQTISDFFQTYQFENLFSKRNTPVAH
ASGFWDYHSFITAAAEYQPHGFGTAGGKLTGQKEVAAFLGHVGSKTSCGY
GVATGGPLAWGLCYNKEMSPSKTYCDDYYKYTYPCTPGVSYHGRGALPLY
WNYNYGKTGEALKTDLLNHPEYLENNATLAFQAAIWKWMTPEKKHLPSAH
DVFVGKWKPTKNDTLAKRVPGFGTTMNVLYGDQVCGKGDDESMNNIVSHY
LYYLDLMGVGREEAGSHECASCAEQLPFNQASASASSTRTRE[redacted]

Endochitinase-*win8*: H1506

Nucleotide:

CTGAA**ATG**AGGTTCTGGGCACTTACAGTTTTATCTTTGTTGTTGTCTCTA
TTACTAGGAGTCTCATCGGATACTGCACAATGCGGATCGCAAGCTGGGAA
TGCCACATGCCCAAACGACCTGTGCTGTAGCAGTGGTGGTTACTGCGGTC
TAACAGTTGCTTACTGTTGTGCAGGCTGTGTAAGCCAATGCCGTAACTGC
TTCTTCACAGAATCGATGTTCGAACAGATGCTACCGAATAGAAACAATGA
CAGCTGTCCTGGAAAGGGATTCTATACGTATGACGCTTATTTTGTGGCCA
CTGAATTCTACCCTGGATTTGGCATGACTGGCGATGATGACACTAGAAAG
AGAGAGCTTGCTGCTTTCTTCGCCCAAACCTCTCAAGAAACTAGTGGACG
GTCGATAATTGGAGAGGATGCTCCATTTACATGGGGATATTGCCTTGTTA
ACGAGCTGAACCCTAATTCTGATTACTGTGATCCAAAAACAAAATCTTCT
TATCCATGTGTAGCCGACTATTATGGCCGAGGTCCTCTACAACTTAGGTG
GAACTACAACTACGGGGAATGTGGAAATTATTTAGGGCAAAATTTATTAG
ACGAGCCAGAAAAGGTGGCAACGGATCCAGTGCTTTCATTCGAGGCAGCA
CTCTGGTTCTGGATGAATCCACATTCAACAGGTGCACCGTCGTGCCATGA
AGTCATCACCGGAGAATGGTCTCCATCAGAAGCGGATATTGAGGCCGGCA
GGAAACCAGGCTTTGGTATGTTAACAAACATTATCACTAATGGCGGTGAA
TGTACTAAAGACGGGAAAACAAGGCAGCAGAATCGCATTGACTACTATTT
GAGGTACTGCGACATGTTACAGGTTGACCCTGGTGACAACCTTTATTGCG
ACAACCAAGAGACATTTGAGGATAATGGACTCTTAAAAATGGTGGGAACC
ATG[redacted]TTTGATGCTAAGGCTATTGCTTGGAATTAGCAAGGACGTCTATG
TACCCCATATAATTAAATAATCCAAGCAATAAATAAATAAATAAAAGGCC
AAGCCATTTGGCTTGTGTTTGTT

Predicted Peptide:

EMRFWALTVLSLLLSLLLGVSSDTAQCGSQAGNATCPNDLCCSSGGYCGL
TVAYCCAGCVSQCRNCFFTESMFEQMLPNRNNDSCPGKGFYTYDAYFVAT
EFYPGFGMTGDDDTRKRELAAFFAQTSQETSGRSIIGEDAPFTWGYCLVN
ELNPNSDYCDPKTKSSYPCVADYYGRGPLQLRWNYNYGECGNYLGQNLLD
EPEKVATDPVLSFEAALWFWMNPHSTGAPSCHEVITGEWSPSEADIEAGR
KPGFGMLTNIITNGGECTKDGKTRQQNRIDYYLRYCDMLQVDPGDNLYCD
NQETFEDNGLLKMVGTM[redacted]

GST: H-1907

Nucleotide:

CCTGTAACTAGCAAATCTA**ATG**GCAAACTCCGGTGACTATATACGAGACC
CACCATTGTCCACAGCAGTGTCTAGAGTCCTAGCTACTCTGATCGAGAAA
GACGTGCCCTTTCACCTCGTTCCCATTGACCTCTCCAAAGGTGAACAGAA
GAAGCCTGAGTACCTCAAGATCCAGCCCTTTGGCCAAGTACCAGCTTTTA
AAGACGAGAGCATCACCCTCTTTGAGTCAAGAGCAATATGCAGGTACATA
TGTGACAAATATGCTGACAAGGGGAACAGGAGCTTATACGGCACAGACAT
CTTATCAAAAGCAAATATAGATCCATGGGTGGAAACTGATGGGCAGACCT
TCGGTTCCCCCAGCCGGGACCTGGTGCCTGAACCTCTATTTAGTAGTGTT
CCCGTAAACCAAGCCTTGATTAAGAAGAACCTAAATAAGTTGGCAAAGTG
CCTGACCTTTACCAACCGAAGCTTGGGACGGACTCAGTTTTTGGCTGGAA
ATGAATTTTCTTT

Predicted Peptide:

MANSGDYIRDPPLSTAVSRVLATLIEKDVPFHLVPIDLSKGEQKKPEYLK
IQPFGQVPAFKDESITLFESRAICRYICDKYADKGNRSLYGTDILSKANI
DPWVETDGQTFGSPSRDLVPEPLFSSVPVNQALIKKNLNKLAKCLTFTNR
SLGRTQFLAGNEFS

LTP: F-1.6.7

Nucleotide:

CCGAGGTGATCTCTGGCATCAACTATGGCGTGGCTGCTGGCCTCCCTTCA
AAGTGTGGTGTATCCATCTCCTACAAAATCAGTCCTTCCACAGATTGCAA
AAGCGTGAAG[redacted]AGAATCGAAGCAGATTATGAAGTATGATAATAAGCTA
GTGCTAGAATAAAAGGGGTGCTCGAGCATGTTTGAGCTAGTTCCCGACGT
CGACGGACTTGGATCAGTCTCTTCTTTCTTATGAGCTTGTGTTTACAG

Predicted Peptide (frame+3):

EVISGINYGVAAGLPSKCGVSISYKISPSTDCKSVK[redacted]

Malate dehydrogenase: H358

Nucleotide:

GTTCTCACCTGCCAGTTTTGCAAAACACCTAGCTATGGAGTCGGTGGCTA
ACCAAAGGATTGCAAGGGTATCAGCTCATCTCCAACCTCCAAATTCCCAG
ATGGAGGAAAGCTGTGTTTTGAAGAGAACTGATTGCAGAGCAAAAGGAGG
AGCGCCTGGGTTCAAAGTGGCTATCTTGGGTGCTGCTGGAGGGATTGGGC
AACCTCTTGCAATGCTAATGAAGATGAATCCTTTGGTCTCAGTTCTTCAC
CTCTATGATGTTGTGAATGCTCCCGGGGTCACTGCTGATATTAGTCACAT
GGACACTGGTGCTGTGGTTCGGGGTTTCCTAGGGCAGCCACAGCTCGAGA
ATGCTCTTACAGGGATGGACCTTGTGATCATACCTGCTGGTGTGCCCAGG
AAGCCTGGGATGACTAGGGATGATCTTTCAAATCATGCTGGGATCGTC

Predicted Peptide:

MESVANQRIARVSAHLQPPNSQMEESCVLKRTDCRAKGGAPGFKVAILGA
AGGIGQPLAMLMKMNPLVSVLHLYDVVNAPGVTADISHMDTGAVVRGFLG
QPQLENALTGMDLVIIPAGVPRKPGMTRDDLSNHAGIV

P450: FTC-5

Nucleotide:

CGAGCTTGAAGTTAAGTTAACTAGAGACAACATCAAGGGATTGACACAGG
ACCTGATTGCTGGAGGAACAGATACCGCTGCTACAATGGGGGATTGGTCA
ATGTCTGAACTCTTGAAAAAGCCCCAGTTATTCAAAAGAGTAACGGACGA
ACTGGATAGAGTTGTTGGGAGAGATAGATGGGTGGAGGAGAAGGACATCC
CACAACTTCCTTACATAGAAGCAATAATGAAAGAGGCAATGAGGATGCAT
CCATCTGCTGTTATGCTTGCACCCCATTTGGCCCTTCAAGATAGCAAAGT
CGGTGGCTATGACATCCCTAAAGGANCCAGAATCTTCATTAACACATGGA
GCATGGGTAGAGACCCTGATCTGTGGGAAGATCCTGAAGATTTCAGGCCA
GAAAGGTTCATCGGTAAAGGAATTGATATTAAAGGACATAACTTCGAGTT
GTTGCCGTTTGGTTCTGGGAGAAGAATGTGCCCTGGCTATCCCCTTGGGA
CGAAGATGATTCTTGTTAGCTTGGCCAACATGCTGCATGGATTCACCTGG
GAATTGCCTCCGGGAATGAAACCACAAGATGTGAAAAGAGATGAGGTTTT
TGGATTGGCAACACAAAGAAAGTACCCAACTGTTGCGGTGGCGAAGNCTN
GACTCCCGCTTCATCTTTACAAT[redacted]CGAATAATATTATTGTAAACATGT
TTAACT

Predicted Peptide (frame+2):

ELEVKLTRDNIKGLTQDLIAGGTDTAATMGDWSMSELLKKPQLFKRVTDE
LDRVVGRDRWVEEKDIPQLPYIEAIMKEAMRMHPSAVMLAPHLALQDSKV
GGYDIPKGXRIFINTWSMGRDPDLWEDPEDFRPERFIGKGIDIKGHNFEL
LPFGSGRRMCPGYPLGTKMILVSLANMLHGFTWELPPGMKPQDVKRDEVF
GLATQRKYPTVAVAKXXLPLHLYN[redacted]RIILL*TCLT

Pop3: F-13

Nucleotide:

CCCAGTTGACTACAAAGAGAGCAGCATACATCCATAGAGAGAAAGAGAAG
AC**ATG**GCAACCAGAACTCCAAAGCTTGTGAAGCACACATTGTTGACTCGG
TTCAAGGATGAGATCACACGAGAACAAATCGACAACTACATTAATGACTA
TACCAATCTGCTCGATCTCATTCCAACCATGAAGAGTTTCAATTGGGGCA
CGGATTTGGGCATGGAGTCTGCGGAGCTAAACCGAGGATACACTCATGCC
TTTGAATCTACATTTGAGAGCAAGTCAGGTTTGCAAGAGTACCTCGATTC
TGCTGCTCTTGCTGCATTTGCAGAAGGATTTTTGCCTACTTTGTCACAGC
GTCTTGTGATAGACTACTTTCTCTAC[redacted]ATGCTCAGGAGTAACGACTTC
GGCCGGGCTATTTCATGG GAATAAAGTAATGTAATGTGCAATAAATGCTG
GTTTTGAACCACTGAATGTTCGTGTCTTGATTTCTTGTTTGTGCTGTGCT
ATGTGAAGGGAGTGCTGCTATTCCTTAAAAATAAAACCCTTGGGGTTGAG
TTGTTGTTTTTCAAAAAAAAAAAAAAAAAGGAATTC

Predicted Peptide:

MATRTPKLVKHTLLTRFKDEITREQIDNYINDYTNLLDLIPTMKSFNWGT
DLGMESAELNRGYTHAFESTFESKSGLQEYLDSAALAAFAEGFLPTLSQR
LVIDYFLY[redacted]

Pop3-like:

Nucleotide:

CTGAATTCGGCACGAGAAAAGGAGTGAGATATTCAAGAGAAATACGTTTA
GAAATCAAGAGAGAGAGAGAGAGAGAGAGAGAGTTAAGACA**ATG**GCCTCG
AAGAAATCTGCAATCGTATTACCTGGTTCAAAGGTGTTGAAGCACATAGT
TTTTGTACGGTTCAATGATGGGATCACTGATGAACAAATTGAGAAATTCA
TAAAAGACTATGCTCATGTCGCCGATGTAGTAGAACCCGTGAAGGGATTT
GGCTGGGGTAAAAACTATCCTGTTCAGCAACTGAACCATGATTACCTTTA
CGGGTTTGAGCTTTCATTTGACAGTCAGGATGCCTTCAATGAGTATCTTC
AGTCTCCTGCTCTCACTGAATTTCATGCCAAGTTTTTGCCTGCCTGTGCA
AGCCGTATGATCATGGACTACTATCTCTTC[redacted]GTCTCTCCAAGGATAAT
GAACCTCTGCTGCCCTGCAATTTTCATTTGTAAATGGAAATATGCTGAAG
TAATAAAGATCAACTCGTGTTGAATGTTTCTTTGGTTAAGTTAAATTGAA
GTGCTGTATATGTTGGAAACCTTTGTGTGGTTCAGGGTTTAACGATCCCT
ATCAATAAACAAATATTTTATGATACTCTGTTTTTCTCGTGCCGAATTC

Predicted Peptide:

MASKKSAIVLPGSKVLKHIVFVRFNDGITDEQIEKFIKDYAHVADVVEPV
KGFGWGKNYPVQQLNHDYLYGFELSFDSQDAFNEYLQSPALTEFHAKFLP
ACASRMIMDYYLF[redacted]

RD22-like:F-2.6.6

Nucleotide:

ACCAAACCAAGATGCAGAAGTATACAATTAAGACCGGAGTAAAGAAGGTG
GCAGGAGACAAATCTGTAGTGTGCCACAAGCAGAACTATGAATATTCCGT
CTTTTACTGCCATGCAACGCAAACCACTAGGGCTTACACGGTTCCCTTGG
AGGGTGATGATGGAACAAAGGCTAAAGCTGTAGCAGTGTGCCACACAGAT
ACGTCAGCATGGAACCCAAAGCATTTGGCTTTCCAGGTGCTCTACGTTAA
GCCAGGNACCGTACCTGC

Predicted Peptide (frame+3):

QTKMQKYTIKTGVKKVAGDKSVVCHKQNYEYSVFYCHATQTTRAYTVPLE
GDDGTKAKAVAVCHTDTSAWNPKHLAFQVLYVKPGTVP

Unknown: F-71

Nucleotide:

CTTCTCTGCTCTCTCTCAGCATCCAAACCCTAACCAGATTTTCTTGAGCA
TCATTCAGCAAGG**ATG**ACAACAGAAGCACCTTTCCGACCAAGGGAGAAGC
TCGTTGAGCACCAGAAATATTTCCAAAGCATTCACAAGCACACATATTTG
AAGGGACCTCTTGATAAGGTTACCTCTGTTGCCATTCCAATAGCATTCGC
AGCCACCTCACTTTTTCTTATTGGGCGAGGGATCTATAACATGTCTCATG
GGATTGGAAAGAAGGAA[redacted]GGAGGCTGTTTGTGTAATGATCACTGTTAT
GTTTTTACTGCTCATGTTTTGAAGGATTATTCGCTTATGCATGTGACCAG
TATTATTTTTAAATTTGTTAATTAATAATTGCATAGTTGCTGGATTGCAG
CTACCAATGGAAGTTTAAACTTCACCATCGATGCCTTTGTTTTCTATTTG
GCTTTTAATGATACATGATTGAATTTCAGGGTTAAAAAAA

Predicted Peptide:

MTTEAPFRPREKLVEHQKYFQSIHKHTYLKGPLDKVTSVAIPIAFAATSL
FLIGRGIYNMSHGIGKKE[redacted]

VSP-*win4.5*: H2339

Nucleotide:

CGAGCGTTAACTTGGTAGTGATGGTGGTTGGGCTGCTGGTTTTGGCTCAG
CAGTCCTTCCAA**ATG**AGTTTGAGAAACCCTGTTGCTGAGACAAACAATTG
CAAAATTGATTTCACTCGTTTAGGGCTTGTCTTAACTTCTGATACCAACG
AAAAGGCCCTTCAAGACTCTGGTCTCTTCACACCTGATGCTGAAACGCCT
TATGTTGACATTGCGGGGAGAAGGTTCCACATTGGGACACTTAATGCTCG
TTATATTGTATATGTTAAGATCGGGGGAAATTCTGTAAATGCTGCCATTG
CTGTGCAAATCCTCTTGAATAGATTCCGTATTCAGGGAATTATTCACTTT
GGTAGTGCTGGGAGCCTTGATAAAGAAAGTATAGTGCCAGGTGATGTTTC
CGTGCCGCTTGCTGTAGCTTTCACTGGAGCTTGGAATTGGAAGAAATTCG
GGTCAGATAAAGGGACGCTGAACTTTGGCGAGTTTAATTATCCAGTGAAC
GGAGAGAACTTGTTGGCTAGCGTAGACTATGATAAAGTAAAATTGTTCTC
TAAAGGACACTCACCGCAGGATGTTTTCTGGTTTCCCAGCACCACATCCT
GGTATAGTGCTGCCACACAAGTGCTTCAGGATTTGGAGTTGAGACAATGC
TACGATAGAGCTTGCCTATCTTCCAAGCCTAAGATTGTGTTTGGAACTAA
CGGCTCTAGTTCTGATTCTTACATTAAAAATAAAGCATATGGAGATTTCC
TTCATAAAGTTTTTAACGTCTCAACTGCCGATCAAGAAAGCGCTGCTGTA
GCTTGGACATCTTTATCAAATGAAAAGCCATTCATCGTGATCCGAGGTGC
TTCCAATGTAGCAGGTGAAGCAAATCCAGGATTTTCGCCTGCTAGCTACT
TGGCCTCCTACAATGCTTTCCTTGCCGCAGCCAAGTTCATTGAGTCAATT
CCTACGCCACGTCTGGCTTGTGAG[redacted]TGGGATTATGTATCTGCTTCTGA
GTCGTTTGAGTGCTATGAAAGAGATCGACGACCGATTGCACTCAAATGAA
CTGAACTGTGTTTTGCCTACTAAATTAAATAAACTCGAATAAAAATGCTA
TGAACTTTCTCC

Predicted Peptide:

MVVGLLVLAQQSFQMSLRNPVAETNNCKIDFTRLGLVLTSDTNEKALQDS
GLFTPDAETPYVDIAGRRFHIGTLNARYIVYVKIGGNSVNAAIAVQILLN
RFRIQGIIHFGSAGSLDKESIVPGDVSVPLAVAFTGAWNWKKFGSDKGTL
NFGEFNYPVNGENLLASVDYDKVKLFSKGHSPQDVFWFPSTTSWYSAATQ
VLQDLELRQCYDRACLSSKPKIVFGTNGSSSDSYIKNKAYGDFLHKVFNV
STADQESAAVAWTSLSNEKPFIVIRGASNVAGEANPGFSPASYLASYNAF
LAAAKFIESIPTPRLACE[redacted]

VSP (PNI288-like): F-55

Nucleotide:

ACGTGCTTGGCTTTCGCTTTCTCTAGGGAAATCA**ATG**GCCTTGGCTGGAT
TGCCAAGTAAAATGTTGGCAGNTGAATTGGCTGTGATGGTGCTTGTGCTA
CTGGCCATGGCACAGCAGTCCATGCAGCTGAGCTTGAGAAGCCCTTTACC
TGAGGTTGTCAAGAAAATCAATGGCAATACAGTGTCTTATTTAGGCATTG
TTGTGAGTTCCTCTGCCAGCGAAGACGCTCTTCTTAATTCTGGGTTCTTC
GTTCCCAGCACACATGTACCTTACATTGACTTAGTCGGAAGAAGATTCAA
CATTGGAAAGATTAAGGACGTTCATGTCGTTGTTGTTAACGTAGGGGGTG
AAATTCCAAATGTAGTTCTAGGCACCCAAGTTCTGTTCGATTTACTTAGC
ATCCGAGGAATCATTCATTTTGGGAGTGCTGGAAGTGTCAGCGATTCGTT
GCGTCTTGGTGATGTTGCTGTGCCAGAGTCTGTTGCCTTCACAGGAAATT
GGGAATGGAAGAGCAACGCGTCAACGAGAGGGGAACTCAAATTCGGAGAC
TTCAATCTTCCACAGAAAGGAGTCAATTCCTTGGGAAGCGCAGATTTTCA
GAAAGTAAAACTGTATACTTCCGGGAACCCATCACAGAACCTTTTGTGGC
TTCCCGTCGATTCAAACTGGCTTGCGGTTGCCTCTGAGCTGCAGGGTTTG
AAGTTGCAAGAGTGTGTAAATGAGATAACCGAGACGAATTGCTTAGAGAA
CACGCCGGAAATCGTCTTCGGAGGAAGGGGCTCCTCTGCCGATATATACC
TTAAGAATGCAGCATATGGCGAATTCCTTGCCAATAGATTCAATGCCACA
TTTGTGGATACATCGAGTGCTGCTGTTGCTTTGGCTTCTTTGACAAATGA
AGTGCCTTATATCTTGTTCCGTGCTATTTCGAATTCGGTAATTCAAGGAA
CATCAGGCCCCAACTCTCACTATTTGGCCACCGCCAACTCTGTCAAAGTT
GCAGTCAAGTTCATTGAGTTGATTGGTAAACCGAACTGGGTGTTCAAGTA
C[redacted]AATCAACTAGTATGCCCTTTCCTCAATAAGGTCATGGTGAAAAGCT
TTATGCTCTATGTCCATGTCATGTACTCAATAATGAAGTGTAATGAGGAG
GGTATTCTCTGTCTTTAACGTTGAAAAAAAAAAA

Predicted Peptide:

MVLVLLAMAQQSMQLSLRSPLPEVVKKINGNTVSYLGIVVSSSASEDALL
NSGFFVPSTHVPYIDLVGRRFNIGKIKDVHVVVVNVGGEIPNVVLGTQVL
FDLLSIRGIIHFGSAGSVSDSLRLGDVAVPESVAFTGNWEWKSNASTRGE
LKFGDFNLPQKGVNSLGSADFQKVKLYTSGNPSQNLLWLPVDSNWLAVAS
ELQGLKLQECVNEITETNCLENTPEIVFGGRGSSADIYLKNAAYGEFLAN
RFNATFVDTSSAAVALASLTNEVPYILFRAISNSVIQGTSGPNSHYLATA
NSVKVAVKFIELIGKPNWVFKY[redacted]